# UNIVERSITY OF LONDON THESIS

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# TUMOUR ANGIOGENESIS IN EPITHELIAL OVARIAN CANCERS.

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**Abstract:**

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy. It is frequently diagnosed late and shows poor prognosis with a 5 year survival rate of 30%. New blood vessel formation (angiogenesis) and new lymphatic vessel growth (lymphangiogenesis) are fundamental events in tumour growth and metastatic dissemination. Despite the existence of established clinical prognostic markers for EOC, there is still a lack of clinically reliable molecular markers for assessing prognosis. In addition, little is known about the molecular events underlying EOC formation and spread.

The aim of the study was to identify additional prognostic parameters in EOC and potentially pre-malignant ovarian lesions and to understand the potential mechanisms of angiogenesis / lymphangiogenesis in EOC formation.

Archival paraffin wax-embedded sections, frozen tissues, serum and fluid samples of pre-malignant ovarian lesions (endometriosis, benign cystadenomas and borderline tumours) and EOC were used to assess molecular changes in EOC and these ovarian lesions compared to normal ovaries. Techniques used included immunohistochemistry, ELISA, and real-time quantitative RT-PCR which were performed to analyse molecular markers of angiogenesis and lymphangiogenesis (VEGF, VEGF-C, VEGFR-1, VEGFR-2, TP and MVD). IGF-1 isoforms involved in cell proliferation, repair and angiogenic regulation were analysed.

Both VEGF and TP expression increased significantly in the formation of endometriotic and borderline lesions. VEGF-C was high in benign cystadenomas and borderline tumours. Serum VEGF levels were higher in EOC compared to premalignant benign ovarian lesions and controls, particularly in clear cell and endometrioid EOC subtypes. There was no correlation between serum VEGF and matched platelet counts. Expression of IGF-1Ea and VEGF increased significantly from normal ovary to EOC, suggesting that VEGF may be under the regulation of IGF-1Ea. This analysis has revealed the molecular interplay of angiogenic and lymphangiogenic pathways in ovarian carcinogenesis.

Dedicated to my late father,

France (Ah Cheng) WONG TE FONG

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Finally, I would like to express my extreme gratitude to my husband Ah Sik, Lam Kam Sang for standing by me relentlessly throughout this time as well as for the support of my mother Vee Chow and sisters (Annick Ah Po and Nadynn Wong Chap Lan), family and friends.

**Declaration:**

I, Lan Fong WONG TE FONG, confirm that the work presented in this thesis is entirely my own. Where information has been derived for other sources, I confirm that this has been indicated in the thesis.

None of the work contained within this thesis has been submitted previously either by myself or by any other unknown, for examination within the University of London or other awarding body.

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**ABBREVIATIONS**

| | |
|---|---|
| aFGF | Acidic fibroblast growth factor |
| AFP | Alpha-feto protein |
| AKT2 | Murine thymoma virus oncogene homolog 2 |
| ANG | Angiopoietin |
| APES | 3-Aminopropyltriethoxy-silane |
| AVD | Average vessel density |
| bFGF | Basic fibroblast growth factor |
| BOT | Borderline ovarian tumour |
| BRCA | Breast and ovarian cancer associated gene |
| BSA | Bovine serum albumin |
| CA-125 | Cancer antigen 125 |
| CAM | Chick chorioallantoic membrane |
| CASA | Cancer associated serum antigen |
| CD-105 | Endoglin |
| CEA | Carcinoembryonic antigen |
| CT | Computerised tomography |
| CV | Coefficient variation |
| Cy | Cystadenoma |
| DFS | Disease-free survival |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonuclease triphosphates |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epithelial/Epidermal growth factor |
| ELISA | Enzyme linked immunosorbent assay |
| Ems | Endometriosis |
| Endo-cc | Endometrioid and clear-cell carcinomas |
| EOC | Epithelial ovarian carcinoma |
| ErbB2 | protein kinase (tyrosine) 2: EGF receptor |
| FA | Focal areas |
| FIGO | Federation Internationale de Gynecologie Obstetrique |
| FGF | Fibroblast growth factor |
| FMS | Colony stimulating factor 1 receptor |
| FSH | Follicle stimulating hormone |
| 5-FU | 5-Fluorouracil |
| g | Gravitational force |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GMCSF | Granulocyte macrophage colony stimulating factor |
| H&E | Haematoxylin & eosin |
| HDMEC | Human dermal micro-vascular endothelial cells |
| HER-2/NEU | same as erbB2 |
| HGF/SF | Hepatocyte growth factor/scatter factor |
| HIER | Heat-induced epitope retrieval |
| HIF | Hypoxia inducible factor |
| HNPCC | Hereditary nonpolyposis colorectal cancer |
| HRP | Horse Radish peroxidase |
| HRT | Hormonal replacement therapy |
| HVD | Highest vessel density |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor binding protein |
| IGF-1R | Insulin-like growth factor receptor |

| | |
|---|---|
| $IgG_2B$ | Immunoglobulin type $G_2B$ |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IMS | Industrial methylated spirit |
| JUN | Fibrosarcoma virus-induced tumour |
| kb | Kilo base |
| kD or kDa | Kilo Dalton |
| KRAS | Kirsten rat sarcoma virus homologue |
| LCM | Laser capture microdissection |
| LYVE-1 | Lymphatic vessel endothelial-receptor 1 |
| LH | Luteinising hormone |
| LREC | Local Research Ethics Committee |
| M-CSF | Macrophage colony stimulating factor |
| MMP | Matrix metalloproteinase |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| Muc | Mucinous |
| MVD | Microvessel density |
| MYB | Myeloblastosis virus homologue |
| MYC | Myelocytomatosis viral oncogene homologue |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD-ECGF | Platelet derived endothelial cell growth factor |
| PDGF | Platelet-derived growth factor |
| PECAM | Platelet-endothelial cell adhesion molecule |
| PIER | Protease induced epitope retrieval |
| PROX-1 | Murine homologue of prospero |
| PTEN | Phosphatase and tensin hmologue |
| qRT-PCR | Quantitative reverse transcription-polymerase chain reaction |
| RAS | Rat sarcoma/erythroleukemia virus-induced tumour |
| RNA | Ribonucleic acid |
| SBC | Streptavidin biotin complex |
| Ser | serous |
| SMA | Smooth muscle actin |
| TATA box | Repeat of T and A nucleotides |
| TBS | Tris buffered saline |
| TGF | Transforming growth factor |
| Tm | Melting temperature |
| TNF | Tumour necrosis factor |
| TS | Thymidylate synthetase |
| TP | Thymidine phosphorylase |
| TSG | Tumour suppressor gene |
| TSP | Thrombospondin |
| UTR | Untranslated regions |
| VCAM | Vascular cell adhesion molecules |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| vHL or VHL | Von Hippel Lindau |
| VIN | Vulval intraepithelial neoplasia |
| vWF | Von Willebrand factor |
| WHO | World Health Organisation |

# CHAPTER 1

# INTRODUCTION

## 1.1 OUTLINE TO INTRODUCTION

This thesis investigates the important role of tumour angiogenesis in the development of epithelial ovarian cancer (EOC). It is widely known that angiogenesis is essential for neoplastic growth and metastasis, and has been the subject of investigation for many years to unravel its mechanism in cancer development and progression. Although traditional histopathological parameters such as recognition of special histological sub-types of carcinoma and grading can sometimes predict the patient's progression, no specific and reliable prognostic / diagnostic factor for EOC has been found. This introduction briefly describes the anatomy, physiology and histology of the ovary, and the changes taking place in the normal ovarian cycle. The nature of EOC and angiogenesis are discussed, and potential factors linked between the pathogenesis of EOC and, the development of blood vessels in tumours are reviewed. Finally, the value of EOC angiogenic diagnostic factors will then be analysed.

## 1.2 THE NORMAL OVARY

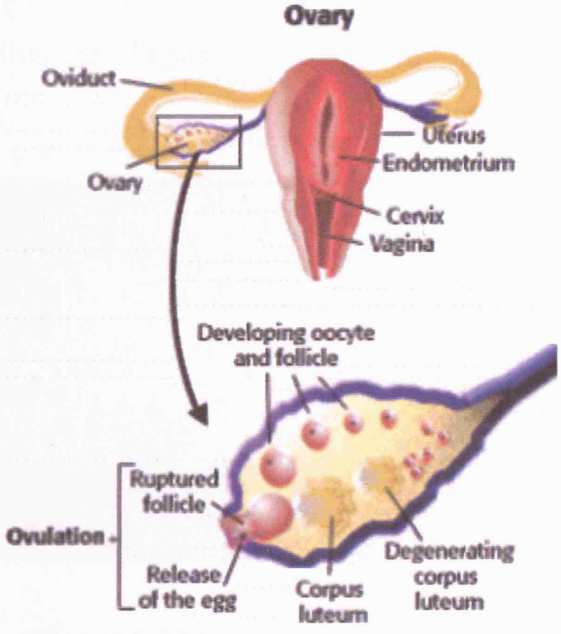



**Figure 1.1 Diagram of the human ovary** (Ref www.biology.arizona.edu)

### 1.2.1 OVARIAN ANATOMY

Figure 1.1 shows the ovaries which are paired, ovoid-shaped organs, whitish-grey in colour which vary in size in different women, and at different times throughout life. In the infant, the ovary is a delicate, elongated structure, with a smooth, glistening surface. The ovary of a neonate contains little stroma and mainly consists of primary follicles. As infancy and childhood progress, increasing numbers of follicles degenerate, and there is a progressive increase in stromal content, up to puberty. During puberty the ovary enlarges, and in the reproductive period it measures approximately 30 x 20 x 10 mm and weighs about 7 g. Post-menopausally, the ovary undergoes rapid regressive changes, becoming wrinkled, white in colour and reduced in size. The ovaries are attached to the posterior leaf of the broad ligament by a fold of the peritoneum known as the mesovarium, through which pass the ovarian vessels and nerves. The part of the ovary between the two layers of the mesovarium is called the hilum. The remainder of the ovarian cortex is covered by a single layer of low columnar epithelium which is in continuity with the mesovarium, under which is a layer of connective tissue, called the tunica albuginea. Figure 1.2 demonstrates the clinical presentation of the ovaries.

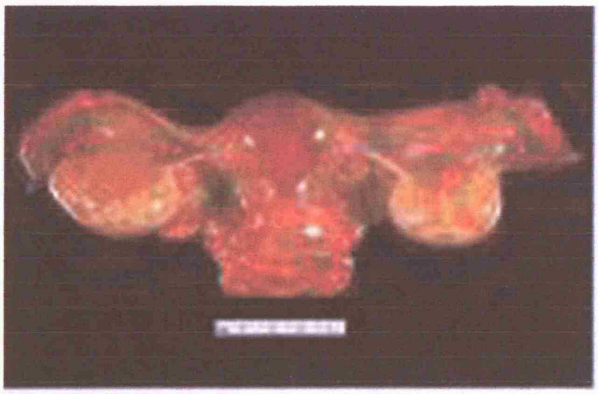


**Figure 1.2 Photograph of the ovaries**

The ovary derives arterial blood from the long, slender ovarian artery, a branch of the abdominal aorta, which arises just below the renal artery. Venous drainage occurs via a plexus of veins (the pampiniform plexus) that drain into the ovarian veins, which lie alongside the ovarian arteries. The right ovarian vein terminates in the inferior vena cava and the left ovarian vein terminates in the left renal vein. Lymphatic drainage occurs via the para-aortic nodes, which lie alongside the origin of the ovarian artery. Some drainage also occurs through the inguinal nodes, via the round ligament and the inguinal canal, and through the contralateral ovary via the uterine fundus.

The nerve supply of the ovary is very well developed and arises from sympathetic (vasoconstrictor) fibres, which surround the ovarian vessels in the infundibulopelvic ligament. These fibres derive from branches of the aortic and renal plexuses and have their pre-ganglionic cell bodies at the level of the $10^{th}$ and $11^{th}$ aortic segment in the spinal cord. Some parasympathetic fibres (vasodilators) from the hypogastric plexus, via the uterine artery, also supply the ovaries.

### 1.2.2 OVARIAN HISTOLOGY

Histologically, each ovary consists of an outer capsule; a peripheral cortex and a central medulla. The capsule consists of an outer covering, the surface epithelium (a simple columnar epithelium derived from the peritoneum, which is often attenuated or missing in adult women) and an inner covering, the tunica albuginea (a protective layer derived from fibrous connective tissue between the germinal epithelium and the ovarian cortex). In adult ovaries, the outer cortex is poorly vascularised and contains many primordial follicles, each consisting of an oocyte (the female gamete) surrounded by flattened stromal cells, termed follicular or

granulosa cells. The cortex is interrupted at the hilum. The inner medulla contains loose connective tissue with abundant blood vessels, lymphatic vessels and nerve fibres (see Figure 1.3).

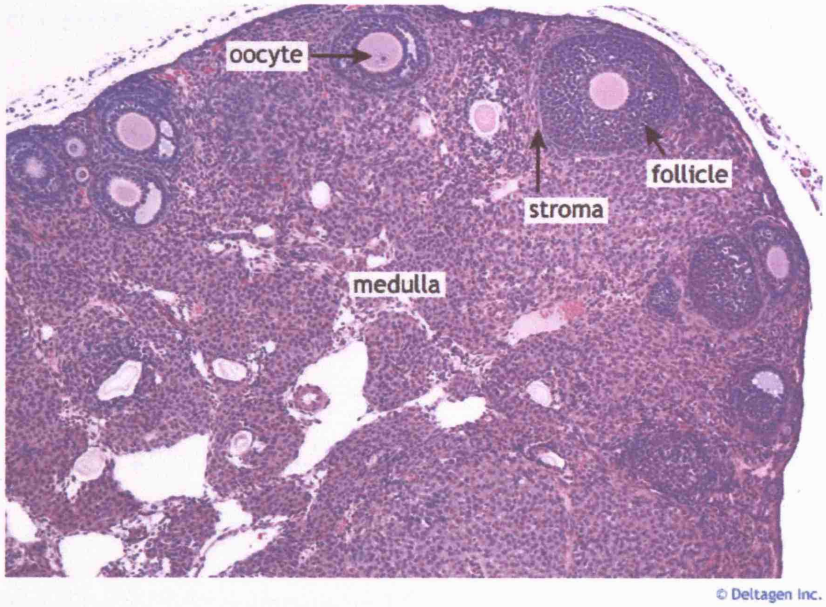



**Figure 1.3 Histological section of normal ovary** (Ref: www.deltagen.com)

In adult ovaries, the cortex contains three types of follicles at various stages of development: primordial (the largest proportion), growing and mature or Graafian. Primordial follicles are present just beneath the tunica albuginea and consist of a single layer of flattened cells. Growing follicles consist of enlarged cuboidal cells that will become stratified. These growing follicles can be subdivided into 2 types: the primary follicles with no cavity and the secondary follicles, which contain small cavities that gradually join up into one large cavity, the antrum, as maturation occurs. The mature or Graafian follicles differ from the growing follicles mainly by their larger diameter (up to 25 mm).

### 1.2.3 OVARIAN PHYSIOLOGY

During each menstrual cycle many primordial follicles are recruited, but usually only one develops fully to become a mature Graafian follicle, which expels its

oocyte. The granulosa cells multiply and secrete follicular fluid. The oocyte with its granulosa layer projects into the follicle. The stromal cells surrounding the granulosa cell layer differentiate into the theca interna (a weak androgen secretor) and the theca externa (no hormone secreting function). Shortly before ovulation, meiosis is completed in the primary oocyte in response to the luteinising hormone (LH) surge. The oocyte casts off the first polar body resulting in the number of chromosomes in the remaining nucleus being reduced from 46 to 23. Thus the primary oocyte and the first polar body each contain a haploid number (23) of chromosomes.

At this stage, the mature follicle is about 20 mm in diameter. At ovulation, this follicle ruptures, releasing the oocyte usually into the fimbriated end of the fallopian tube. The follicle collapses and the granulosa cell lining becomes folded. Cells in the granulosa layer and theca interna enlarge and begin secreting steroids, forming the theca lutein cells. A corpus luteum develops and projects from the surface of the ovary (see Figure 1.1). It can be recognised by the naked eye by its crinkled outline and yellow appearance. Its cells secrete oestrogen and progesterone. If the ovum is not fertilised, the corpus luteum degenerates in about 10 days. A small amount of bleeding occurs into its cavity, the cells undergo degeneration and a corpus albicans - a dense connective tissue scar - is formed, and eventually removed by macrophages. If fertilisation does occur, the corpus luteum enlarges. It is maintained for 6 months and gradually degenerates thereafter. In addition to oestrogen and progesterone, it produces relaxin, a hormone that facilitates the pelvic opening to enlarge during parturition. The menstrual cycle is regulated at the endocrine level by the cyclical release of follicle stimulating hormone (FSH), LH and prolactin from the anterior pituitary.

Pituitary FSH stimulates follicular growth during the first half of the menstrual cycle. Growing follicles produce oestrogen, whose high mid-cycle level exerts negative feedback on the pituitary gland reducing FSH production. This causes the LH surge, which directs follicular maturation, stimulates ovulation and controls the formation and maintenance of the corpus luteum. The corpus luteum produces both oestrogen and progesterone. Progesterone inhibits LH production, causing the corpus luteum to degenerate after approximately 10-12 days unless fertilisation occurs. If fertilisation does occur, the developing placenta produces chorionic gonadotrophin that will maintain the corpus luteum in the absence of LH.

The inhibins are members of the TGF-β family of growth factors that are secreted in the female principally from the ovarian follicles in the female and Sertoli cells in the male. Following oophorectomy or after menopause with the depletion of ovarian follicles, the circulating level of inhibin becomes undetectable. In contrast, inhibin levels are elevated in women with ovarian cancer in particular those with granulosa cell tumours and those with the mucinous subtype of EOC. Clinical investigations of inhibin to detect ovarian cancer have shown that it complements CA-125, an established marker of EOC. Robertson et al (2007) have shown that the two markers together have detected up to 95% of ovarian cancers with 95% specificity.

## 1.3 BENIGN OVARIAN TUMOURS

### 1.3.1 PATHOLOGY

Benign ovarian tumours may arise from any of the 3 main elements that constitute the ovary; surface epithelium, oocytes and mesenchymal stromal elements. The

pathology of these benign tumours and their major subtypes are listed in Table 1.1.

| Benign ovarian tumours | Major subtypes | Cell origin |
|---|---|---|
| **Non-neoplastic cysts** | Follicular cyst<br>Luteal cyst<br>Endometriotic cyst<br>Epithelial inclusion cysts | Developing follicle<br>Corpus luteum<br>Abnormally sited endometrium<br>Surface epithelium |
| **Benign epithelial tumours** | Serous cystadenoma<br>Mucinous cystadenoma<br>Endometrioid cystadenoma<br>Brenner tumour<br>Benign clear cell tumour<br>Adenofibroma (cystadenofibromas) | Surface epithelium |
| **Benign germ cell tumours** | Cystic teratoma<br>Mature solid teratoma | Germ cells |
| **Benign sex-cord stromal tumours** | Fibroma, thecoma<br>Granulosa cell tumour<br>Sertoli-Leydig cell tumour | Sex-cord stromal cells |

**Table 1.1 The pathology of benign ovarian tumours.**

1.3.1.1 NON-NEOPLASTIC CYSTS

These are cysts that are formed in the ovary from either physiological structures or as non-neoplastic pathological conditions. Some are asymptomatic. Although they may occur in any pre-menopausal woman, these cysts are particularly likely in young women. They may also occur in premature female infants and in women with trophoblastic disease.

**1.3.1.1A Follicular cysts:** These are enlarged cystic unruptured Graafian follicles which may form by failure of normal atresia in a non-dominant follicle. Lined by

granulosa cells with an outer layer of theca cells, they are the most common benign ovarian lesions. They are usually unilateral and may secrete oestrogen or be relatively quiescent. Because of this, the symptoms are variable. Follicular cysts are particularly likely in patients undergoing ovulation stimulation, when they are commonly multiple. They usually disappear during menstruation, before another menstrual cycle begins. If not, they will shrink in about one to three months. Women who do not ovulate regularly can develop **multiple follicular cysts**. This is a disorder in which the ovaries are enlarged and contain many small cysts. This can be caused by polycystic ovarian syndrome (PCOS). Symptoms of PCOS include irregular menstrual periods, infertility, obesity and increased body hair.

**1.3.1.1B Luteal cysts (luteinised follicular cysts):** Luteal cysts are similar to follicular cysts, except that the thecal layer is luteinised. They occur when, instead of degenerating when implantation of the embryo fails to occur, the corpus luteum survives and grows. These may present with intraperitoneal haemorrhage.

**1.3.1.1C Endometriotic cysts:** Endometriotic cysts (endometriomas) develop in women who have endometriosis, a condition in which tissue from the lining of the uterus (endometrium) grows in other areas, such as on the fallopian tubes, the ovaries and on other organs outside the pelvis. Because endometrial tissue is sensitive to hormones, it bleeds monthly in the same way as the uterine lining which may cause it to form a growing cyst in the ovary which becomes filled with a thick, brown altered blood, so called 'chocolate cysts'. These cysts can be very painful because there is an inflammatory and fibrotic reaction to the haemorrhage.

**1.3.1.1D Epithelial inclusion cysts**: These cysts probably form by inclusion of islands from the surface 'germinal' epithelium of the ovary in the areas where ovulation has occurred.

1.3.1.2 BENIGN EPITHELIAL OVARIAN TUMOURS

The majority of ovarian neoplasms, both benign and malignant, arise from the ovarian surface epithelium. They are essentially mesothelial in nature, deriving from the coelomic epithelium, which also forms the peritoneum. These neoplasms may differentiate along a variety of pathways including endocervical (mucinous cystadenomas), endometrial (endometrioid), tubal (serous) pathways or uroepithelial (Brenner) lines respectively. Although benign epithelial tumours tend to occur at a slightly younger age than their malignant counterparts, they are most common in women over 40 years.

**1.3.1.2A Serous cystadenomas:** These are the most common benign epithelial tumours and are frequently detected in women aged between 35–55 years. The cysts are lined by cuboidal or columnar ciliated epithelium resembling that of the fallopian tube, and the cells secrete thin, watery fluid. They occasionally contain calcified granules known as *psammoma bodies*. Serous cystadenomas are uni- or multilocular and are bilateral in about 10% of cases.

**1.3.1.2B Mucinous cystadenomas:** These constitute 15-25% of all ovarian tumours and are the second most common benign epithelial tumour. Like serous cystadenomas, benign mucinous tumours occur most frequently between the ages of 35 and 55 years. They can grow to a considerable size and are usually

multilocular with a smooth inner surface. They are usually unilateral and are lined by tall columnar cells, each of which has a basal nucleus and cytoplasmic mucin. Mucin, which is generally thick and glutinous, is constantly secreted into the cyst so that its wall becomes tense. Occasionally these cysts may rupture releasing mucinous cells, which may become attached to the peritoneum and omentum leading to an intraperitoneal accumulation of mucin (*pseudomyxoma peritonei*- being occasionally malignant). In addition to mucinous cells resembling the endocervix, some tumours have a lining resembling the intestinal epithelium.

**1.3.1.2C Endometrioid cystadenomas:** Most endometrioid tumours are malignant and will be discussed later (see section 1.4.2.1C). However, benign endometrioid cysts do occur but have to be differentiated from endometriosis by the absence of surrounding endometrial stroma.

**1.3.2.1D Ovarian endometriosis:** This is a common benign, oestrogen dependent disorder, highly associated with clear cell carcinoma and endometrioid carcinomas. It is recognised by the presence of small red, blue and brown spots and patches associated with scars and dense fibrous adhesions. Microscopically, endometriosis is characterised by the presence of benign endometrial-type glands and stroma. Ovarian endometriosis is associated with extensive stromal haemorrhage, dense fibrosis of the stroma and cyst formation. Severe disease results in extensive pelvic adhesions and distortion of the pelvic anatomy, which can lead to pelvic pain, dyspareunia (pain on intercourse), dysmenorrhoea (painful periods) and infertility. In women with mild disease and no symptoms, the risk that pain will develop subsequently is very low (Moen et al, 2002). For women

with pain, surgery commonly provides temporary relief, although symptoms recur in up to 75% of women within 2 years (Candiani et al, 1991; Kuohung et al, 2002; Olive et al, 2002), and further surgery is needed in many cases (Olive et al, 2002). It has a prevalence of 6-10% in the general female population; in women with pain, infertility, or both the frequency is 35-50% (Sensky and Liu, 1980; Houston, 1984).

**1.3.1.2E Brenner tumours:** Brenner tumours are formed of islands of transitional cell epithelium. They are rare, accounting for only 1–2% of all ovarian tumours and are bilateral in 10–15% of cases. Usually small, these tumours are composed of nests of epithelium, resembling the transitional cell epithelium of the urinary tract, in a dense fibrotic stroma, giving a largely solid appearance. The epithelial component may be benign, borderline or malignant, but the vast majority are benign. No such tumours are studied in this thesis.

**1.3.1.2F Benign clear cell tumours:** These are only rarely benign. The typical histological appearance is of cells with clear cytoplasm and/or 'hobnail' cells arranged in mixed patterns.

**1.3.1.2G Adenofibromas or cystadenofibromas:** These are epithelial cystic epithelial tumours with a fibrotic stromal component that produces bulbous intracystic papillary structures covered by a thin epithelium.

1.3.1.3 BENIGN GERM CELL TUMOURS

These tumours are among the most common ovarian tumours seen in women less than 30 years of age. Only 2–3% are malignant but in the under 20's, this proportion may rise to a third. As the name suggests, benign germ cell tumours arise from totipotential germ cells and may contain elements of all three germ layers (embryonic differentiation).

**1.3.1.3A Mature cystic teratomas (dermoid cysts)**: Benign dermoid cysts are the commonest benign germ cell tumours. They result from irregular differentiation into mature tissues of which skin is the commonest component. They account for around 40% of all ovarian neoplasms. They occur in all age groups but are more common in young pre-menopausal women. They are bilateral in about 11% of cases, seldom large and often asymptomatic. Rupture, however is very painful as it may release sebaceous material from the skin sebaceous glands into the surrounding tissues.

**1.3.1.3B Mature solid teratomas:** These rare tumours also contain mature tissues but are less likely to include the skin.

1.3.1.4 BENIGN SEX-CORD STROMAL TUMOURS

These tumours represent 4% of benign ovarian tumours and occur at any age from pre-pubertal children to elderly postmenopausal women. They arise from the specialised mesenchyme of the ovary, particularly from the granulosa and thecal cells, as well as from the spindle cells of the ovarian stroma. They may be hormone producing.

**1.3.1.4A Fibromas:** These tumours are most frequent around 50 years of age. They are thought to be derived from stromal cells and have features in common with thecomas. They are hard, mobile and lobulated with a glistening white cut surface. Less than 10% are bilateral.

**1.3.1.4B Thecomas:** Thecomas (theca cell tumours) are very rare. They are lobulated, solid tumours with a well-circumscribed margin and a 'yellow' surface macroscopically. The tumours are composed of plump spindle cells containing fine lipid droplets, which make the cytoplasm appear foamy. The tumours often secrete oestrogens and because of this, they may be associated with endometrial hyperplasia or endometrial carcinomas.

## 1.4 MALIGNANT OVARIAN TUMOURS

Ovarian malignant tumours have a similar incidence to malignant uterine tumours but ovarian malignancy is responsible for more cancer deaths because the paucity of symptoms in the early stages means that they usually present at a later stage. At the time of clinical presentation, 60–70% are stage 3 or 4, and tumour cells have often spread throughout the peritoneal cavity.

### 1.4.1 INCIDENCE, EPIDEMIOLOGY AND SURVIVAL

According to Cancer Stats (Cancer Research UK Statistics, September, 2005), ovarian cancer accounts for 5% of all cancers among women in the UK (Office for National Statistics, Cancer Statistics Registrations, England and Wales, 2005) and it accounts for 6% of all cancer deaths in women, which is far more than all the other gynaecological cancers combined. In 2000, the Cancer Research UK statistics estimated that there were about 6,700 new cases and 4,400 deaths per

year. The UK has one of the highest incidences of ovarian cancer in Europe. In the industrialised world, only Japan shows an exceptionally low incidence rate with 3-6 newly diagnosed ovarian cancers/100,000 per year. Low incidence rates are also observed in Africa and Asia (World Cancer Research Fund, 1997). Ethnic factors play an important role and influence the incidence rates of ovarian cancer. In the European Union, incidence rates are highest in Denmark, Austria, Sweden, and Ireland, intermediate in Germany, and lowest in Greece, Portugal, Spain, Italy and France (Arbeitsgemeinschaft Krebsregister, 1997).

Most ovarian malignancies are of epithelial origin. These are rare before the age of 35 years but the incidence increases with age to a peak in the 50-70 year old age group. Just under half occur in women aged 45–65 years (Quinn et al, 2001). Most epithelial tumours are advanced at diagnosis so only 36% of women with ovarian cancer are alive at 5 years (Cancer Stats, September 2005). Eventually, 75-80% of women with ovarian cancer will die from their disease. Only 3% of ovarian malignancies are seen in women younger than 35 years and the vast majority of these are non-epithelial types, such as germ cell tumours.

### 1.4.2 PATHOLOGY

Ovarian tumours are named according to the type of cellular differentiation shown and whether the tumour is benign or malignant. In addition, there are those, which while having some of the features of malignancy lack any evidence of stromal invasion. These are called borderline tumours or tumours of low malignant potential, atypical proliferating tumours.

The most commonly used classification of ovarian tumours was defined by the World Health Organisation (WHO; Scully, 1999). It is a morphological

classification based on cell types and patterns of the tumour compared to tissues normally present in the ovary. This classification categorises the ovarian tumours with regard to their derivation from coelomic surface epithelial cells, germ cells and mesenchyme (the stroma and the sex cord). Epithelial ovarian tumours, which are the majority of malignant ovarian tumours, are further grouped into histological types as follows: serous, mucinous, endometrioid, clear cell, transitional cell tumours (Brenner tumours), mixed epithelial tumour, undifferentiated carcinoma, and unclassified carcinoma (see Table 1.2). In this thesis, it is this group of malignant epithelial ovarian tumours that are the main focus of the study; however, non-epithelial malignant ovarian tumour types (germ cell and sex-cord stromal) will also be described, but briefly.

| **Ovarian tumours** | **Histological type** |
|---|---|
| **Epithelial tumours** | (borderline or malignant)<br>Serous<br>Mucinous<br>Endometrioid<br>Clear cell<br>Transitional (Brenner)<br>Mixed epithelial tumour<br>Undifferentiated carcinoma<br>Unclassified tumour |
| **Sex-cord stromal tumours** | Granulosa stromal cell tumour<br>Theca cell tumour<br>Sertoli-Leydig cell tumour |
| **Germ cell tumours** | Dysgerminoma<br>Yolk sac tumour<br>Immature teratoma |
| **Metastatic tumours** | Krukenberg tumour |

(This table is only for malignant tumours as benign tumours are in Table 1.1).

**Table 1.2 A simplified version of the WHO histological classification of ovarian tumours.**

1.4.2.1 EPITHELIAL OVARIAN CARCINOMA (EOC)

Epithelial ovarian tumours constitute about two-thirds of all ovarian neoplasms and an even greater proportion of over 85% of the malignant ovarian tumours (Koonings et al, 1989), occur predominantly in adults, with the malignant forms generally appearing later in life. They are classified according to the predominant pattern of differentiation of the tumour cells (Table 1.2).

**1.4.2.1A Serous cystadenocarcinomas**: They are by far the commonest primary carcinoma, accounting for 40% of all cases, and in over half the cases, they are large and often bilateral. The cystic cavity tends to be filled by complex branching papillary structures. These structures are covered by columnar cells, which are crowded and dysplastic. The cells are stratified and form solid sheets. Essential for the diagnosis of malignancy is evidence of invasion of the stroma by tumour cells; indeed, the tumour may breach the capsule of the ovary forming papillary outgrowths on the surface. Histologically, they resemble the epithelium of the fallopian tube. They contain clear, serous fluid. 20% present as stage I; 5-year survival is 15-30%.

**1.4.2.1B Mucinous cystadenocarcinomas:** They comprise about 12% of malignant tumours of the ovary. They are characterised by glands and cysts lined by epithelial cells usually containing abundant intracystoplasmic mucin with basal nuclei. The cells are usually columnar, but the nuclei are larger and more pleiomorphic than the equivalent benign tumours. The cells are crowded and show

increased mitotic activity. Mucinous tumours are amongst the largest tumours of the ovary and may reach an enormous size. Evidence of malignancy is demonstrated by the presence of invasion of tumour cells into the stroma. Histologically, they resemble either the endocervical epithelium or intestinal mucosal epithelium. 50% are stage I, therefore 5-year survival is 40-45% for all stages of mucinous cystadecarcinoma.

**1.4.2.1C Endometrioid carcinomas:** These adenocarcinomas may arise from pre-existing ovarian endometriosis and they account for 2-4% of all ovarian tumours and 20% of all malignant EOCs. The tumour spreads by local invasion through the myometrium and via lymphatics to iliac and para-aortic lymph nodes. Histologically, they resemble the usual adenocarcinoma of the endometrium and are solid masses with a soft, firm, or fibrous consistency. They are often predominantly cystic and contain soft or firm nodular masses. The cysts are usually filled with chocolate-coloured fluid. Ovarian endometrioid carcinomas are bilateral in 30% of cases and 15% are associated with endometrial carcinomas; they have a 5-year survival of 40-45%.

**1.4.2.1D Clear cell carcinomas:** They comprise 5–10% of all ovarian carcinomas in Western countries (Scully et al, 1998) but they are more common in Japan (Kaku et al, 2003). Bilaterality is rare and they are thought to be a variant of endometrioid carcinomas, characterised by the presence of cells with clear cytoplasm rich in glycogen, and hobnail cells. The latter are characterised by apical nuclei that protrude into the lumens of tubules beyond the cytoplasmic limits of the cells.

**1.4.2.1E Transitional cell (Brenner) tumours:** Transitional cell tumours represent 1-2% of all ovarian tumours and are characterised by nests and columns of epithelial cells, most or all of which are of transitional type resembling urothelium cells. Mucin-filled cells are present in varying amounts in one-third of the cases. Also, malignant–appearing squamous cells and mucinous cells are common in Brenner tumours, and calcification is noted in most cases.

**1.4.2.1F Mixed epithelial tumours:** They occur when various subtypes of the surface epithelium stromal category are present altogether. The WHO limits their identification to those neoplasms in which one or more components other that the predominant component account for at least 10% of the tumour on microscopic examination (Scully et al, 1998).

**1.4.2.1G Undifferentiated carcinoma**: An undifferentiated carcinoma is one that is anaplastic and shows no differentiation or contains only rare, minor areas of differentiation according to the WHO (Silva et al, 1991; Scully et al, 1998). It can grow rapidly and is frequently widely spread at diagnosis with a 5-year survival of 15%.

**1.4.2.1H Borderline epithelial tumours:** Borderline epithelial ovarian tumours or tumours of low malignant potential are ovarian neoplasms with intermediate histopathological features between benign lesions and malignant tumours. Borderline tumours are confined to the ovaries and have a better prognosis than carcinomas. The histological diagnosis of a borderline tumour is made by the presence of features of malignancy such as nuclear atypia and an increase in

mitotic activity, multilayering of neoplastic cells and formation of cellular buds, but no stromal invasion. If small, foci of invasion are identified then the tumour may be designated as microinvasive. About 10% of all epithelial ovarian tumours are borderline tumours, of which 30% are of mucinous type, followed by the serous type. Other histological types of borderline tumour are rare. They often occur in reproductive-age women. Because of the generally benign behaviour of these tumours, their management has become progressively more conservative, allowing women to maintain their fertility.

*Borderline serous tumours*: These tumours contain many of the cytological and architectural features of serous cystadenocarcinomas but they lack stromal invasion. The cells are stratified into several layers, forming tufts. They are also characterised by complex papillary growths that may contain spherical, concentrically laminated, calcified bodies in their stroma. The cells exhibit mild to marked nuclear atypia, and mitotic figures are present, but neither of these features is as pronounced as in serous carcinomas. The overall prognosis depends on the presence and type of accompanying peritoneal lesions. Sometimes referred to as 'peritoneal deposits,' these are more likely to represent 'field change' tumours similar in type to the ovarian tumours. If the peritoneal lesions are invasive, the prognosis is worsened and adjuvant therapy is required.

*Borderline mucinous tumours*: These show many of the cytological features of mucinous adenocarcinomas but without invasion of the stroma. Their prognosis is better than that of the mucinous cystadenocarcinomas. In fact, there are two types of mucinous borderline tumours (Rutgers and Scully, 1988). The more common type is characterised by intestinal-type epithelial cells and a second type by endocervical-type cells. The latter is often associated with endometriosis and has

papillae lined by slightly to moderately atypical epithelial cells that may contain abundant cytoplasmic mucin. In contrast, intestinal-type mucinous borderline tumours may either lack papillae or have branching papillae lined by atypical epithelium with variable goblet cells and other intestinal cell types. Some apparent mucinous borderline tumours have been shown to be metastatic from intestinal, especially appendiceal lesions. These may be associated with pseudomyxoma peritonei (Young et al, 1991).

The epidemiological risk factors for borderline tumours are similar to those of EOCs. However, the mean age at diagnosis of women with borderline tumours is about 45 years, which is 10 years younger than that of EOCs (Aure et al, 1971; Hopkins et al, 1987; Harris et al, 1992). The annual incidence is higher among whites than among non-whites, being 26.2/million and 16.5/million respectively, in the state of Washington, USA (Harlow et al, 1987). The treatment for borderline tumours is generally total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy. Adjuvant therapy for borderline tumours confers no survival benefit. However, if fertility is desired, a unilateral oophorectomy appears not to have a detrimental effect on survival rates.

### 1.4.2.2 NON-EPITHELIAL MALIGNANT OVARIAN TUMOURS

Non-epithelial tumours constitute about 10% of all malignant ovarian tumours. Due to their rarity and sensitivity to intensive chemotherapy, it is especially appropriate to correctly diagnose them and to refer these patients for specialist care.

**1.4.2.2A Sex-cord stromal tumours:** Granulosa and theca cell tumours are the most common sex cord stromal tumours. They often secrete steroid hormones, particularly oestrogens, which can cause postmenopausal bleeding in older women and sexual precocity in prepubertal girls. The hormones may cause endometrial hyperplasia or even endometrial carcinoma.

**1.4.2.2B Germ cell tumours:** These are uncommon ovarian tumours accounting for 2-5% of all primary malignant ovarian tumours. Originating in the germ cells, 90% occur in young women less than 30 years of age and are bilateral in 15% of cases.

*Yolk sac tumours:* They are the second most common malignant germ cell tumours, making up 10-15% of all malignant germ cell tumours reaching a high proportion in children. They are characterised by the presence of a variety of patterns, with a loose vacuolated network of microcysts lined by flat cells of mesothelial appearance. The most characteristic feature is the endodermal sinus (Schiller-Duval body).

*Immature teratomas:* These tumours comprise about 1% of all ovarian teratomas. They are solid and often malignant. They may contain a wide variety of tissues (may contain areas of bone and cartilage) but the immature element is nearly always neural in type.

1.4.2.3 METASTATIC TUMOURS

The ovary is a common site for metastatic spread, particularly from the breast and gastrointestinal tract. A well-known example is the so-called *Krukenberg tumour* in which there is infiltration of the ovary by mucin-secreting adenocarcinoma of a

'signet ring' pattern; such tumours are usually derived from stomach or colon, and metastasise to the ovary by either transcoelomic or lymphatic spread. Tumours from other parts of the genital tract, including uterus and cervix, may also involve the ovaries. Both breast tumours and lymphomas can also metastasise to this site.

### 1.4.2.4 CLINICAL STAGING

The staging of ovarian cancer is used to assess the size of the cancer and the extent to which the lesion has spread throughout the body (whether it is localised to the ovary only or has already invaded the pelvis and lymph nodes). It is defined by the FIGO (Federation Internationale de Gynecologie Obstetrique) system, which is summarised in Table 1.3.

The stage distribution of serous carcinoma differs from that of other histological types of ovarian carcinoma. Serous carcinomas are bilateral in about two-thirds of all cases but in only 13% of Stage I cases. Serous carcinoma is predominantly found in stage III or IV. On the contrary, clear cell (63%), endometrioid (48%) and mucinous (71%) carcinomas tend to remain confined to the ovary (stage I). In clear cell carcinomas, combined figures from two large studies have revealed that 64% of them are stage I and 86% stage I or stage II at the time of exploration (Aure, 1971).

---

**Stage I** - Growth limited to the ovaries.

| | |
|---|---|
| IA | Growth limited to one ovary;<br>no ascites present; no tumour on external surface; capsule intact. |
| IB | Growth limited to both ovaries;<br>no ascites; no tumour on external surfaces; capsule intact. |
| IC | Tumour either Stage IA or IB but tumour on surface of 1 or both ovaries;<br>or with capsule ruptured; or with ascites containing malignant cells or with positive peritoneal washings. |

**Stage II** - Growth involving 1 or both ovaries with pelvic extension.

IIA Extension and/or metastases to the uterus or fallopian tubes, or both.
IIB Extension to other pelvic tissues.
IIC Tumour either Stage IIA or IIB but tumour on surface of one or both ovaries;
or with capsule ruptured; or with ascites containing malignant cells or with positive peritoneal washings.

**Stage III** - Growth involving 1 or both ovaries with peritoneal implants outside the pelvis or positive retroperitoneal or inguinal nodes. Tumour limited to the true pelvis but with histologically proven malignant extension to the small bowel or omentum. Superficial liver metastases equals stage III.

IIIA Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
IIIB Tumour with histologically confirmed implants on abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes are negative.
IIIC Abdominal implants greater than 2 cm in diameter or positive retroperitoneal or inguinal nodes.

**Stage IV** - Most advanced stage of ovarian cancer. Growth involving 1 or both ovaries with distant metastases. If pleural effusion is present there must be positive cytology to allot a case to stage IV. Parenchymal liver metastasis equals stage IV.

**Table 1.3 The International Federation of Gynaecology and Obstetrics (Federation Internationale de Gynecologie et d'Obstetrie, FIGO) staging system for primary ovarian carcinoma (1986).**

1.4.2.5 GRADING OF EOC

EOC are given a grade according to the WHO classification, though it is not a unanimously accepted system (Barber et al, 1975; Day et al, 1975; Swenerton et al, 1985). It is based on the histological level of differentiation of the tumour cell population and is on a scale of 1-3 (see Table 1.4).

| Grade | Histological level of differentiation |
|---|---|
| 1 | well-differentiated |
| 2 | moderately differentiated |
| 3 | poorly / undifferentiated |

**Table 1.4 Definition of the different grades of EOC.**

### 1.4.3 AETIOLOGY OF EOC

The aetiology of ovarian cancer is poorly understood but some risk factors are recognised and there is considerable interest in trying to determine the pathogenesis of epithelial ovarian cancer.

#### 1.4.3.1 RISK FACTORS

**1.4.1.3A Family History:** Hereditary or familial ovarian cancer only accounts for 5% to 10% of ovarian cancers, the vast majority being sporadic. The risk of ovarian cancer depends on the number of affected first- and second degree relatives, as well as their age at diagnosis with ovarian or breast cancer. This applies to relatives on both the maternal and paternal side. There are at least 2 distinct groups of individuals with a hereditary predisposition to ovarian cancer for which pedigree analysis suggests autosomal dominant transmission with variable penetrance. Families with BRCA1 or BRCA2 gene mutations are associated with a predisposition for breast and ovarian cancer. On the other hand, there are those individuals who have ovarian cancer as part of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (Lynch II syndrome) due to mutations in DNA mismatch repair genes (Lynch and Smyrk, 1996). In addition there may be other as yet unidentified genes, which predispose to familial ovarian cancer.

**1.4.1.3B Reproductive factors, infertility and fertility drugs:** While young age at menarche is a frequently quoted risk factor for ovarian cancer, recent studies do not confirm this (Franceschi et al, 1991; Purdie et al, 1995). However, late menopause may be associated with a trend towards a slightly higher risk of ovarian cancer (Franceschi et al, 1991). An increased ovarian cancer risk among

nulliparous women is consistently reported (Negri et al. 1991). After correcting for the effect of voluntary nulliparity, studies have found infertility to be a significant risk factor for ovarian cancer (Bristow and Karlan, 1996).

**1.4.1.3C Environmental, dietary and host factors:** In epidemiological studies, numerous additional variables have been found to be associated with ovarian cancer with weak or mixed degrees of correlation, such as the use of talc in genital hygiene (Cramer et al, 1999), tobacco smoke (Doll et al, 1980), radiation exposure (Pettersson et al, 1985), psychotropic medication (Harlow et al, 1998), the mumps virus (Cramer et al, 1983), high-level physical activity (Mink et al, 1966), dietary factors such as lactose or galactose (Risch et al, 1994) and caffeine consumption (La Vecchia et al. 1984). While one study suggested an excess risk of endometrioid ovarian cancer associated with post-menopausal women using oestrogen (Weiss et al, 1992) most studies assessing the effect of post-menopausal hormone use on ovarian cancer have not found any association (Whittemore et al, 1992; Purdie et al. 1995). In addition, a prior history of pelvic inflammatory disease (Risch and Howe, 1995), PCOS (Schildkraut et al, 1996) or endometriosis have been associated with an increased risk of ovarian cancer, the latter especially for endometrioid and clear cell types (Brinton et al, 1997).

1.4.3.2 PROTECTIVE FACTORS

**1.4.3.2A Reproductive life:** Numerous studies have found parity to be protective against ovarian cancer. Overall, multiparous women have shown a risk reduction as high as 40-60% compared to nulliparous women (Risch et al, 1994; Hankinson et al, 1995). It is estimated that each delivery confers a 16-22 % risk reduction

(Adami et al, 1994; Risch et al, 1994; Hankinson et al, 1995), independent of the age at first pregnancy. Pregnancies which result in either spontaneous or induced abortions lead to minimal or no significant change in ovarian cancer risk (Negri et al, 1991; Risch et al, 1994). However, lactation has been found to be associated with a slight additional reduction in the risk of ovarian cancer (Rosenblatt et al, 1993; Risch et al, 1994).

**1.4.3.2B Pharmacological agents:** There is a strong protective association between oral contraceptive use and ovarian cancer. According to Rosenblatt and Howard, the risk of ovarian cancer decreases with increasing duration of oral contraceptive use. Women have their risk reduced by 40, 53 and 60% with oral contraceptive use for 4, 8 and 12 years, respectively. This protective effect of oral contraceptives appears to persist after discontinued use (Schlesselman, 1995). Other results by Gross and Schlesselman study suggest that nulliparous women who use oral contraceptives for 5 years or more can reduce their risk of ovarian cancer to that of parous women who have never used oral contraceptives (Gross and Schlesselman, 1994). In addition, there is an additive effect between parity and oral contraceptive use reflected in the observation that women who have 2 children and have used oral contraceptives for at least 5 years have a 70% risk reduction for ovarian cancer (Franceschi et al, 1991). A compatible 60% risk reduction with 6 or more years of oral contraceptive use has also been observed in high-risk patients with BRCA1 and BRCA2 mutations (Narod et al, 1998).

Patients diagnosed with borderline ovarian tumours who also suffer from infertility are willing to expose themselves not only to conservative surgery but also to infertility treatments, despite explanations regarding the controversy

concerning the association between infertility drugs and ovarian tumours. Ovulatory stimulants; these so-called fertility drugs include bromocriptine, clomiphene citrate, gonadotropins and gonadotropin-releasing hormone have been used in the treatment of ovulatory disorders; in *in vitro* fertilisation, to produce eggs for retrieval and sometimes in donor insemination to regulate timing of ovulation. As a fertility treatment, ovulatory stimulants increase the risk of multiple pregnancies and may cause a serious condition known as hyper-stimulation syndrome.

**1.4.3.2C Tubal ligation and hysterectomy:** In several cohorts and case control studies, tubal ligation and hysterectomy have been associated on average with a 67% risk reduction for ovarian cancer. This protective effect appears to last for at least 20-25 years after surgery (Hankinson et al, 1993; Miracle-McMahill et al, 1997).

1.4.3.3 MODELS OF OVARIAN CARCINOGENESIS

The aetiology of ovarian cancer appears to be multifactorial, with genetic, environmental, and endocrinological factors directly or indirectly related to its pathogenesis. Based on the epidemiological and pathological studies mentioned above, there are several theories regarding the aetiology of ovarian cancer which are not mutually exclusive. Low parity and late menopause increase the risk of ovarian cancer, while pregnancy and lactation, as well as the use of oral contraceptive pills, confer protective effects. These observations support the 'Incessant Ovulation Theory' of Fathalla that incessant ovulation, with its repetitive disruption and repair of the ovarian surface epithelium, may lead to a

higher probability of spontaneous mutations and thus increase the risk of ovarian cancer (Fathalla, 1971). In addition, there appears to be a direct correlation between the number of ovulatory cycles and the risk of ovarian cancer (Bernal et al, 1995), further supporting the concept that a person's ovulatory history is a strong index of ovarian cancer risk. This model does not explain why infertility, often due to hypo- or anovulation, is associated with an increased risk of ovarian cancer. This led to the 'Gonadotrophin Theory' suggesting that excessive gonadotrophin stimulation of the ovary contributes to an increased ovarian cancer risk (Cramer and Welch, 1983). However, more recent studies of serum gonadotrophin and androgen levels question the aetiological role of elevated serum gonadotrophin and suggest that it is elevated androgen levels which are associated with an increased risk (Helzlsouer et al, 1995). Another theory – the Pelvic Contaminants Theory – has proposed that the exposure of the ovary to pelvic contaminants and carcinogens may increase the risk of ovarian cancer. Observations based on the use of talc in genital hygiene, suggested an increased risk of ovarian cancer with talc usage and a decreased risk with tubal ligation (Parmely and Woodruff, 1974).

### 1.4.4 CLINICAL ASPECTS OF EOC

One of the most frustrating problems in the management of ovarian cancer is the lateness of presentation. The ovary is the only organ in the abdominal cavity not to have a peritoneal covering, which allows it to hang freely. It is also supplied with nerve fibres. It is the combination of these properties that allow the ovary to grow quite large without the patient being aware. Despite the delay in diagnosis, all malignant ovarian tumours generally tend to manifest in a similar manner, with

an insidious onset and the development of vague, non-specific symptoms such as abdominal swelling, abdominal pain and discomfort, weight loss, poor appetite, change in bowel habit, dyspnoea, back pain, dysuria, vaginal bleeding, fatigue or fever.

### 1.4.4.1 SCREENING AND DIAGNOSIS

Benign ovarian tumours grow silently, and often undetected for some years. They do not generally cause pain, but if large may cause discomfort. They rarely affect menstrual function. Benign cysts that are fluid-filled are not likely to be malignant. Those that are solid or a mixture of fluid-filled and solid usually require further evaluation using a combined vaginal and rectal examination to determine if any malignancy is present. If a cyst is suspected, these following procedures are performed:

- A pregnancy test: a positive pregnancy test may suggest that the cyst is luteal.
- Pelvic ultrasound: this is a painless procedure in which a wand-like device (transducer) is used to send and receive high-frequency sound waves through the pelvic area, creating an image of the uterus and ovaries on a video screen. An ultrasound scan helps to look for features, which may suggest malignancy such as protrusions inside the cyst, whether the cyst is multilocular, whether there is neovascularisation, and if there is ascitic fluid in the pouch of Douglas.
- Laparoscopy: using a laparoscope, which is a slim, lit instrument inserted into the abdomen through a small incision, the clinician can visualise the ovaries and remove the ovarian cyst.
- Cytology: In some cases, women may notice a swelling in the abdomen or fluid collecting around their abdomen. This collection of fluid is called ascites and can

cause bloating and discomfort. Using a cannula, this fluid is drained and a sample is removed and sent for cytology where it will be examined to see if there are any malignant cells present. If a pelvic mass is found or ovarian tumour is suspected, further investigations are required to differentiate between benign and malignant tumours and to determine the extent of the disease. These investigations include the use of tumour markers and imaging techniques.

- Tumour markers: Common tumour markers that are currently in use are CA125 (cancer antigen 125), alpha-feto protein (AFP) and carcinoembryonic antigen (CEA). CA125 is a tumour-associated antigen, which is the most useful tumour marker to detect ovarian cancer. Its level is raised in up to 80% of women with epithelial tumours (Bast et al, 1983) and is a good serum marker for monitoring the disease course in EOC (Bast et al, 1983); however, the CA125 blood test has its limitations. Its level can also be raised in many non-malignant conditions such as endometriosis, pelvic inflammatory disease, adenomyosis and pregnancy. It is not recommended as a general screen. Furthermore, other malignant tumours can increase the CA-125 levels such as endometrial, pancreatic, breast, colon, lung cancer, and lymphoma. Also, the values can sometimes be normal in the presence of ovarian tumour deposits. Like CA125, AFP and CEA are both helpful in monitoring treatment and determine recurrence of EOC. They also have their limitations; AFP is more likely to be raised in germ cell tumours such as yolk sac than in EOC and CEA is more indicative of bowel tumour than ovarian tumour. Other existing tumours markers are CA19.9, macrophage colony-stimulating factor, placental alkaline phosphatase, galactosyl transferase, cancer associated serum antigen (CASA) and OVX1; they are all are raised in 30-70% of women with EOC. These tumour markers when used in combination with CA125 can

improve its sensitivity and specificity but are not sufficiently specific when used in isolation (Menon, et al, 2002).

- Imaging techniques: Several imaging techniques are available for the assessment of suspected ovarian cancer or a pelvic mass, and the detection of ascites. These techniques include ultrasonography, with or without Doppler, computerised tomography (CT) scanning, magnetic resonance imaging (MRI), radio-nucleotide imaging and radioimmunoscintigraphy. They all aid in establishing diagnosis as well as in determining the extent of the disease spread and the resectability of more advanced ovarian tumours. Imaging can detect more than 90% of ovarian lesions but no technique alone can accurately predict histology. Tumour size, wall thickness and internal cyst architecture are the commonly used criteria to characterise a primary lesion. However, none of these imaging techniques will detect small peritoneal metastases. The most accurate method for assessing lymph node involvement is biopsy at the time of surgery. Lymphangiography is accurate; however, it has its limitations too as it cannot detect micrometastases and false-positive results are frequent.

1.4.4.2 TREATMENT

**1.4.4.2A Benign cyst:** The treatment for an ovarian cyst depends on the age of the patient, the type and size of the cyst, and the symptoms. Different types of cysts often have similar symptoms. They may look the same with ultrasound. For that reason, the clinician may watch the cyst for several weeks to see if it disappears on its own, without further treatment. Sometimes low-dose oral contraceptive pills are prescribed to stop the body from releasing ova and that allow time for the cyst

to 'involute' on its own before the body resumes its regular cycle. If the cyst does not involute, or if it grows larger, then laparoscopy or laparotomy is considered.

**1.4.4.2B EOC:** The main treatment of EOC is to remove surgically as much tumour as possible and then follow this up with chemotherapy. In the UK, a specialised multidisciplinary team within a Cancer Centre is responsible for the treatment of ovarian cancer. There are two ongoing randomized trials that are evaluating the benefit of adding bevacizumab to carboplatin and paclitaxel in the first line setting: GOG 218 and MRC ICON7. In GOG 218, patients receive bevacizumab in addition to carboplatin and paclitaxel compared with another group of patients receiving carboplatin and paclitaxel alone. This trial also evaluates the impact in overall survival of five concurrent cycles of bevacizumab to six cycles of carboplatin and paclitaxel when compared with six cycles of carboplatin and paclitaxel in women with newly diagnosed stage III and IV epithelial and peritoneal primary caner. MRC ICON7 has a very similar approach, however it recruits patients with ovarian cancer in high-risk early 9FIGO stage I or IIA clear cell or grade 3 carcinoma) and advanced stage (FIGO stage IIB or greater, all grades and all histological subtypes) ovarian cancer (Rosa et al, 2007).
*Surgery* is performed to remove all visible tumours if possible. This is achievable in the majority of stage I cases and in some stage II cases by performing a total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy. In women with stage IA disease that want to conserve their fertility, unilateral oophorectomy with adequate staging may be safe. However, in women with advanced EOC, removal of the entire tumour is often impossible. Because of the diffuse spread of the tumour throughout the peritoneal cavity and retroperitoneal

nodes, microscopic deposits will persist in almost all cases, even when all macroscopic tumours appear to have been excised. Thus, while surgery alone may be curative in many stage I cases, additional therapy is essential for most of the other stages.

*Chemotherapy*: For many years, cyclophosphamide was the standard chemotherapeutic drug; this was then superseded by platinum-based drugs (cisplatin, carboplatin), either as a single agent or in combination with others such as cyclophosphamide or doxorubicin. All these drugs have toxic effects, which limits the dose, and should be administered in a Cancer Centre setting by a medical oncologist. Almost 50% of patients respond to these regimens for stage III disease and whilst the 5-year survival rate is approximately 30%, the average time to relapse is 2 years. Recently, a new group of drugs, the taxanes, derived from the Pacific yew tree, have shown promising response rates and are now used as front line drugs in combination with either cisplatin or carboplatin. Mean response rates of 3 years have been obtained in trials and these drugs are now been seen as the most effective treatment available.

*Other treatments:* Currently radiotherapy is not recommended for the adjuvant treatment of EOC, although it may have a palliative role in recurrent EOC. There is evidence that some ovarian tumours demonstrate a response of 8-14% to hormonal therapies such as tamoxifen, progestational agents, gonadotrophin-releasing hormone analogues and combined oestrogen/progestin preparations (Schwartz et al, 1998). Moreover, tamoxifen has been shown *in vitro* to delay the development of resistance to cisplatin or platinum of ovarian cancer cells (McClay etal, 1994; Benedetti et al, 2001). Hormonal replacement therapy (HRT) is also used increasingly in premenopausal women with EOC whose ovaries have been

removed as replacement hormones. To date, the most widely used therapeutic anti-oestrogen is tamoxifen, but this drug is a mixed agonist/antagonist. It is a fairly good antagonist in the mammary gland and effectively inhibits the growth of oestrogen-responsive breast cancer cells, but it is an agonist in the endometrium and bone (Brooks et al, 1992). Its differential effects seen in breast and endometrium may be explained, in part, by the fact that tamoxifen has an antagonist effect on both oestrogen –receptors α and β (Paech et al, 1997).

Growing tumours require a new blood supply to maintain the supply of nutrients and prevent necrosis (Folkman 1995). The angiogenic process in cancer is maintained by several growth factors released by tumour and stromal cells. Of primary importance among these is VEGF, which stimulates endothelial cell proliferation and vascular permeability. In the uterus as well as in other tissues such as the ovary, oestrogen up-regulates VEGF expression and this may implicate this steroid hormone in the regulation of tumour angiogenesis; interestingly, tamoxifen also upregulates this growth factor in the uterus (Hyder et al, 1996).

## 1.5 MOLECULAR BIOLOGY OF OVARIAN CANCER

In recent years, investigation of the biology of ovarian cancer has identified aberrant, dysfunctional expression and / or mutation of a variety of different classes of genes. This includes oncogene overexpression, amplification or mutation, aberrant tumour suppressor gene (antioncogene) expression or mutation and the inappropriate expression of cytokines, and growth factors and/ or the cellular receptors for these molecules as well as inappropriate expression of adhesion molecules. Figure 1.4 demonstrates the six hallmarks essential for

carcinogenesis, as described by Hanahan and Weinberg (Hanahan and Weinberg, 2000).

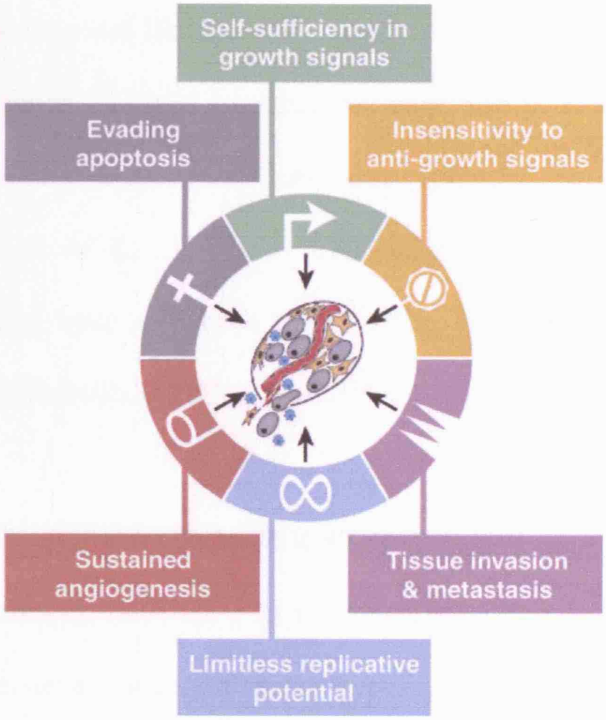



**Figure 1.4 The six hallmarks of cancer**

(Ref Hanahan and Weinberg, 2000 with permission from Elsevier).

### 1.5.1 ONCOGENES

Oncogenes are generally accepted as being dominantly acting genes at the cellular level, which can induce neoplastic transformation. They can be activated by point mutation, amplification or gene rearrangements, leading to a constitutive production of gene products (proteins and messenger RNA; mRNA). In the pathogenesis of ovarian cancer, oncogenes involved are *HER2/Neu, MYC*, *FMS* (colony stimulating factor 1 receptor), *KRAS2* (Kirsten rat sarcoma virus homologue), *AKT2* (murine thymoma viral oncogene homolog 2), *JUN* and *MYB*. At the cellular level, these oncogenes can be subdivided according to their biological role and cellular location (Baker et al, 1990; Bishop et al, 1991). *HER2/Neu* also called *ErbB-2,* involved in ligand binding, was found to be over-

expressed both at the gene and protein level in one-third of ovarian cancers (Slamon et al, 1989). Its expression is associated with poor prognosis (Berchuck et al, 1990). Other oncogenes like the *RAS* family representing the inner membrane proteins and involving in signal transduction, have also been implicated in EOC and are more frequent in mucinous than serous tumours (Enomoto et al, 1991). Oncogenes such as *MYC, JUN* and *MYB*, which are nuclear transcriptional regulatory proteins, have also been described in one-third of ovarian cancers (Baker et al, 1990; Schreiber and Dubeau, 1990).

### 1.5.2 ANTI-ONCOGENES (TUMOUR SUPPRESSOR GENES)

During the last decade, since the *P53* tumour suppressor gene was found to be important in the development of many types of human tumours, numerous publications have outlined its mutation in about 30-60% of EOC. The *P53* gene, located at 17p13.1 is most often mutated on exon 5 and 7 in ovarian cancer (Marks et al, 1991; Kohler et al, 1993; McManus et al, 1994, Allan et al, 1996). The presence of mutations in exon 6 and 8 have also been found, albeit less frequently (Milner et al, 1993; Liu et al, 1994; Allan et al, 1996). The product of the *P53* gene is a nuclear phosphoprotein that can be expressed by normal cells and which plays a role in the regulation of cellular growth and development; however it is overexpressed when mutated (Hollstein et al, 1991; Levine et al, 1991; Marks et al, 1991). The loss of normal p53 function, because of mutation or deletion of the wild type *P53* gene, is often associated with a malignant phenotype. Mutation of *P53* can result in the dominant transformed phenotype, as the expression of a mutant form of *P53* results in the dysfunction of normal p53 protein, preventing the formation of functional DNA-binding. Gottlieb and

colleagues demonstrated that the *P53* gene can be induced by tumour necrosis factor (TNF)-α, together with the induction of cell death by apoptosis (Gottlieb et al, 1994), and therefore, suggested a mechanism by which TNF-α induces tumour cell death may involve the upregulation of tumour cell *P53* gene expression.

### 1.5.3 GROWTH FACTORS AND CYTOKINES

Growth factors and cytokines have been shown to play important roles in the development of cancer (Aaronson, 1991; Cross and Dexter, 1991), including ovarian cancer (Malik et al, 1991; 1992; Martinez-Maza and Berek, 1991; Mills et al, 1992). Among the several growth factors that have been identified to play a role in ovarian cancer are transforming growth factor (TGF)-α / β, and epidermal growth factor (EGF; Mills et al, 1992; Bast et al, 1992; Berchuck et al, 1992). For example, TGF-β may represent a step in the progression of ovarian carcinoma functioning as an autocrine inhibitory factor in normal ovarian epithelial growth which is lost with malignant change (Bartlett et al, 1992). Various cytokines that play important roles in ovarian cancer are TNF-α, interleukin-1 (IL-1), macrophage / monocyte colony stimulating factor (M-CSF) and IL-6 (Malik and Balkwill, 1991; Martinez-Maza and Berek, 1991; Bast et al, 1992; Berek et al, 1992). IL-1 and IL-6 have been shown to enhance the proliferation of some ovarian cancer cell lines (Bast et al, 1992; Wu et al, 1992), and M-CSF, TNF- α, IL-1and IL-6, are produced by ovarian cancer cells.

## 1.6 ANGIOGENESIS

Neovascularisation, the formation of new blood vessels, can be considered as two distinct biological processes: vasculogenesis and angiogenesis. Vasculogenesis

refers to the *de novo* formation of blood vessels during embryogenesis. Angiogenesis is defined as the stimulation of growth of new vascular endothelial cells and the development of new vessels from pre-existing vessels (Folkman, 1971). It is a crucial event in many proliferative processes such as embryonic development, wound healing and reproductive functions (including ovarian cycling) in the adult (Folkman, 1995). Angiogenesis is also implicated in the pathogenesis of a variety of disorders: proliferative retinopathies, age-related macular degeneration, rheumatoid arthritis, psoriasis and tumorigenesis (Folkman, 1995). In endocrine glands, angiogenesis serves as a unique exchange role for secretory products between interstitial fluid surrounding the parenchymal cells and the plasma. Endothelial cells of endocrine glands frequently display fenestrae, which are highly permeable to fluid and small solutes, thus facilitating bi-directional transport (Palade et al, 1979).

### 1.6.1 TUMORIGENESIS IS ANGIOGENESIS DEPENDENT

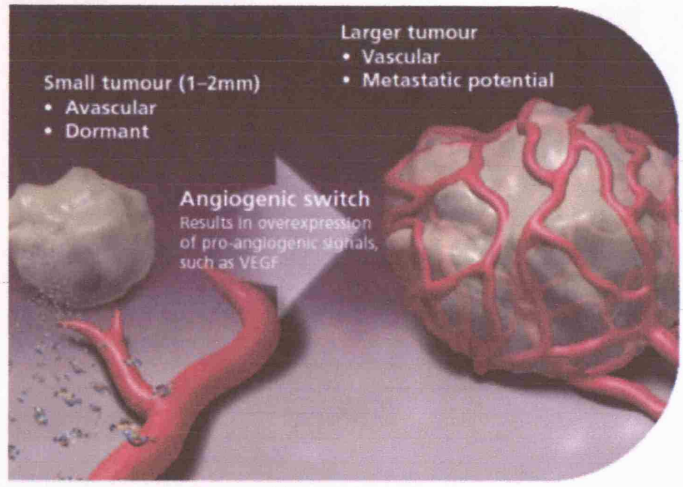
It has been found experimentally that tumours implanted into isolated perfused organs failed to develop; however, if the same tumours were implanted into donor mice, within 6 mm of blood vessels, these tumours induced angiogenesis and also grew rapidly and metastasized (Gimbrone et al, 1972; 1973). Folkman proposed that, without angiogenesis, the growth of solid tumours cannot proceed beyond 1-2 mm because tumour proliferation is severely limited by nutrient supply to, and waste removal from, the tumour into the surrounding medium by the process of diffusion (Folkman, 1971). New capillary blood vessels, derived from pre-existing capillaries or venules must hence be elicited from surrounding host tissue in what it is termed the process of 'tumour angiogenesis' or neovascularisation. By

definition, neovascularisation is therefore a prerequisite for tumour cells to spread by shedding into the circulation; the newly formed, immature, and leaky capillaries to help the process of metastasis because their basement membranes are fenestrated, allowing greater accessibility for stray tumour cells (Folkman et al, 1989). However, in the absence of vascular support, tumours may become apoptotic or even necrotic (Holmgren, 1995).

### 1.6.2 THE ANGIOGENIC SWITCH

The ability of tumours to induce and sustain angiogenesis is thought to be the consequence of two major types of event (Bouck *et al*, 1996; Hanahan and Folkman, 1996). The first is a gain-of-function event in which certain angiogenic growth factors stimulators are induced or unregulated in tumour cells. These include basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF) and angiopoietin-1, among a number of others. The second major change is a loss-of-function event, namely, down-regulation of various endogeneous angiogenesis inhibitors, such as thrombospondin-1 (TSP-1) and interferon (IFN)-α/β. A combination of these two processes can result in local excess in tumours of angiogenesis stimulators over inhibitors, and thus, 'trip' the angiogenic switch at some point during tumour progression (Bouck *et al*, 1996; Hanahan and Folkman, 1996). The switch concept (Figure 1.5) describes the process whereby angiogenesis is activated (Hanahan and Folkman, 1996), i.e., when adult endothelial cells that are usually quiescent, are stimulated to enter the cell cycle and develop into new capillaries. It involves an extracellular matrix, all of which are capable of releasing factors influencing the angiogenesis mechanism. Also, the process involves the stimulation by different proangiogenic growth

factors (stimulators) and reduction in inhibitors of angiogenesis. It is the net balance of stimulators and inhibitors of angiogenesis that determines the final angiogenic phenotype of the tumour.




*The angiogenic switch (from Bergers and Benjamin 2003).*

**Figure 1.5 Angiogenic switch** (Ref: www.avastin-info.com)

### 1.6.3 THE ANGIOGENIC CASCADE

Angiogenesis is a multistep process involving both the endothelium and the extracellular matrix (Risau, 1997). After a stimulatory signal (such as a specific growth factor), activated endothelial cells release proteases (such as plasminogen activator), which lead to the degradation of the extracellular matrix surrounding the vessel (Bicknell, 1997) followed by endothelial migration and proliferation. The endothelial cells are reorganised into a tubular structure, followed by fusion with other newly formed vessels, leading eventually to an anastomosing network (Risau and Flamme, 1995). Figure 1.6 demonstrates the angiogenic cascade.

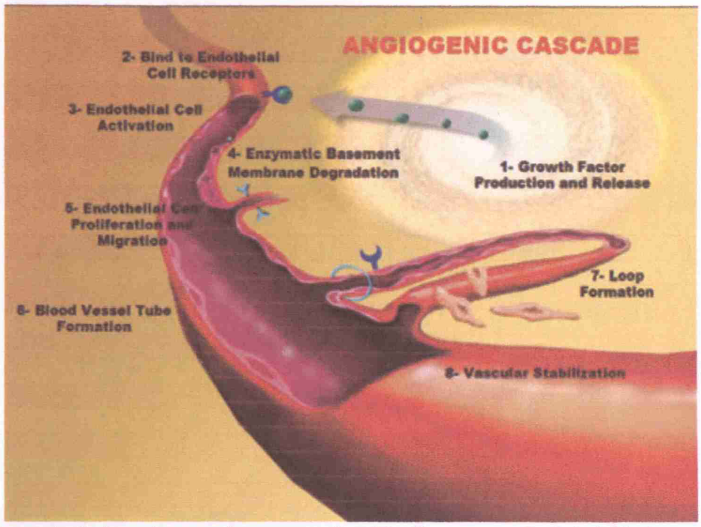



**Figure 1.6 Angiogenic cascade** (Ref: www.thehighlights.com)

### 1.6.4 ANGIOGENIC FACTORS

In recent years, much progress has been made in the identification of several proangiogenic and inhibitory stimuli of angiogenesis (Table 1.5). Among these regulators of angiogenesis, VEGF is widely distributed and has been shown to play a coordinated role in endothelial cell proliferation and assembly of the vessel wall in a variety of normal and abnormal states (Ferrara et al, 1999; Yancopoulos et al, 2000). Five members of the VEGF family have now been identified, in addition to 2 members of the angiopoietin family and at least 1 member of the ephrin family of regulators (Yancopoulos et al, 2000); these are thought to work in a complementary and coordinated manner to form functional vessels (Gale et al, 1999).

| **Angiogenic stimulators** | **Angiogenic inhibitors** |
|---|---|
| Vascular endothelial growth factor (VEGF) | Thrombospondin-1 (TSP-1) |
| Platelet-derived endothelial cell growth factor / thymidine phosphorylase (PD-ECGF/TP) | Platelet factor 4 |
| Insulin-like growth factor (IGF-1) | TGF- β |
| Fibroblast growth factors (basic FGF, acidic FGF) | TNF- α |
| Transforming growth factor-α/β (TGF- α/β) | bFGF soluble receptor |
| | Angiostatin |

| | |
|---|---|
| Platelet-derived growth factor (PDGF) | Prolactin |
| Epidermal growth factor (EGF) | IFN - α/β |
| Tumour necrosis factor- α (TNF- α) | Endostatin |
| Hepatocyte growth factor/scatter factor (HGF/SF) | Vascular endothelial cell growth |
| Granulocyte macrophage colony stimulating factor | inhibitor |
| (GMCSF) | Vasostatin |
| Angiopoietin 1 and angiopoietin 2 | Meth-1 and Meth-2 |
| Interleukin-8 | Interleukin-1, 12 |
| Placental growth factor | Placental proliferin-related protein |
| Matrix metalloproteinase (MMP) | Anti-thrombin III |
| Ephrin family | Tissue inhibitors of MMP |
| Tissue selective angiogenic stimulators, e.g. | |
| endocrine gland-derived VEGF | |
| Integrin α5β3, α5β5 | |
| Proliferin | |

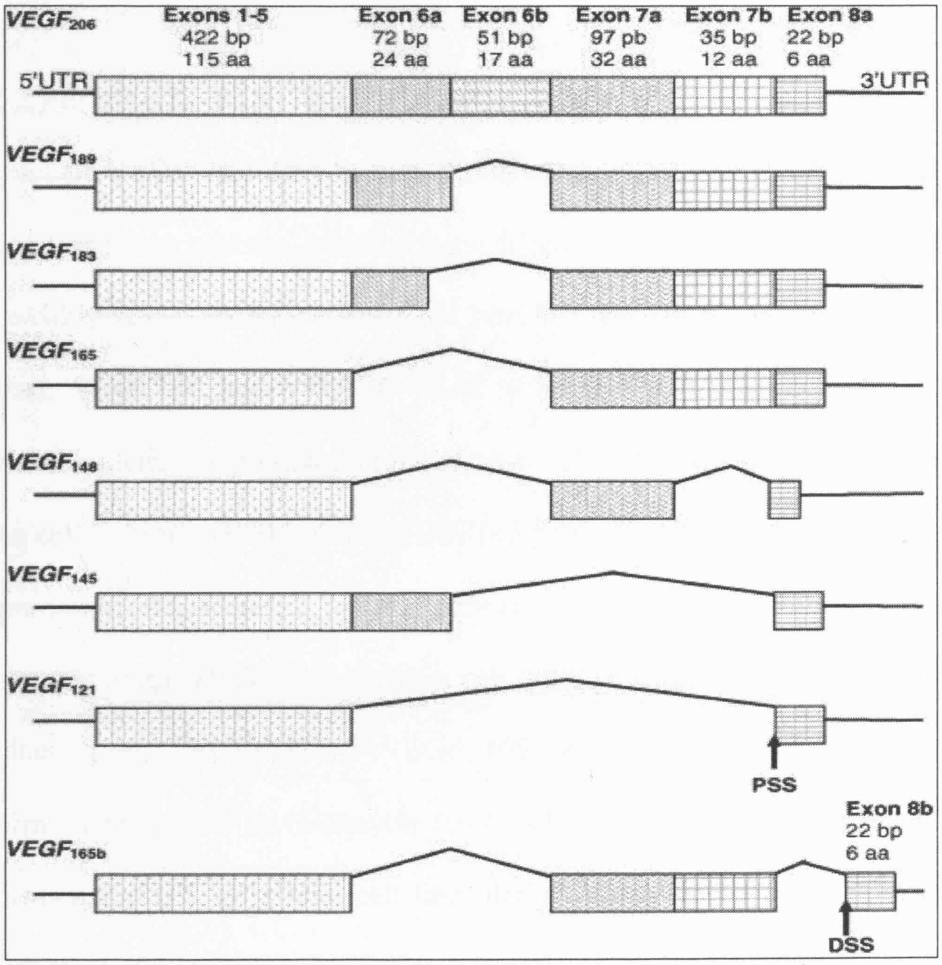
**Table 1.5 Some common mediators of angiogenesis.**

### 1.6.5 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF (also termed as VEGF-A) was first identified in 1983 (Senger et al, 1983), from the conditioned medium of a guinea-pig tumour cell line. It is a potent and very specific mitogen for vascular endothelial cells and is also known as vascular permeability factor, VPF, because it was initially recognised for its ability to increase microvessel permeability (Dvorak et al, 1995). Biochemically, VEGF is a disulfide-bonded dimeric glycoprotein of 34 to 45 kDa (Ferrara, 1999). It is constituted of two chains that are arranged in an antiparallel way, covalently linked by two disulfide bridges between cys-51 and cys-60 (Muller et al, 1997).

1.6.5.1 VEGF ISOFORMS

The human VEGF gene is situated on chromosome 6p21.3 (Vincenti et al, 1996) and is organised in eight exons, separated by seven introns and its coding region spans approximately 14 Kb (Houck et al, 1991; Tischer et al, 1991). Figure 1.7 shows the reported model for human VEGF pre-mRNA that generates the alternative splicing variants (Woolard et al, 2004). Exon 3 and 4 contain VEGFR-1 and VEGFR-2 binding sites, respectively, and exon 6 and 7, heparin binding domains.




(UTR, untranslated region; PSS, proximal splice site; DSS, distal splice site).

**Figure 1.7 Alternative splicing variants of the human VEGF pre-mRNA.** (Ref: Woolard et al, 2004).

To date, in human, at least eight VEGF isoforms (VEGF121, VEGF145, VEGF148, VEGF165, VEGF165b, VEGF183, VEGF189 and VEGF206) are generated by alternative splicing of a single VEGF mRNA (Ribeiro et al, 2006). VEGF isoforms differ by the presence or absence of sequences encoded by exons 6 and 7, whereas the amino acids encoded by exons 1-5 and 8 are conserved in all isoforms (Tischer et al, 1991). VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exons 6a, 6b, 7a and 7b of the VEGF gene (Figure 1.7).

1.6.5.2 PROPERTIES OF THE VEGF ISOFORMS

Despite all VEGF isoforms having an identical signal sequence, they exhibit different secretion patterns, which suggest different physiological roles. VEGF121 is a weakly acidic protein; it does not bind heparin and is freely diffusible. In contrast, VEGF189 and VEGF206 bind to heparin with high affinity and are almost completely sequestered in the extra-cellular matrix and to a lesser extent, on the cell surface. VEGF165 and VEGF145 present intermediate properties; they are predominantly secreted, but a significant fraction remains bound to the cell surface and extra-cellular matrix. Most cell types produce several VEGF variants simultaneously, VEGF121 and VEGF 165 being the most highly expressed isoforms (Ferrara and Davis-Smyth, 1997). VEGF145 is one of the main VEGF isoforms expressed by several cell lines derived from carcinomas of the female reproductive system, reaching levels comparable with VEGF165 (Poltorak et al, 1997) while the VEGF 206 isoform is a very rare isoform that has been, so far, detected in a human fetal liver cDNA library (Houck et al, 1991) and in human

mast cells stimulated with calcium ionophore and phorbol ester (Grutzkau et al, 1998).

1.6.5.3 VEGF FAMILY

VEGF is the founding member of a family of dimeric glycoproteins, which can interact with different receptors to induce endothelial mitogenesis. Other members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factors (PlGF-1 and PlGF-2; Veikkola et al, 2000; Yancopoulos et al, 2000) and a recently identified tissue-specific endothelial growth factor-endocrine gland derived vascular endothelial growth factor, EG-VEGF (LeCouter et al, 2001). The receptors to which the several members of the VEGF family bind are shown in Table 1.6. All these VEGFs contain an approximately 100 amino acid VEGF homology domain, characterised by the precise spacing of 8 cysteine residues. Mice lacking VEGF-B are normal and fertile, but have small hearts, suggesting that VEGF-B may play a role in coronary artery development (Joukov et al, 1997). VEGF-C, due to its ability to bind the lymphatic specific receptor VEGFR-3, has been implicated in lymphangiogenesis - lymphatic development (Olofsson et al, 1999). Like VEGF-C, to which it is structurally related and also lymphangiogenic, VEGF-D is an endothelial cell mitogen and interacts with VEGFR-2 and VEGFR-3. (Yancopoulos et al, 2000). VEGF-E, encoded by the orf virus, induces angiogenesis through an interaction with VEGFR-2 (Meyer et al, 1999). PlGFs are, as its name implies, strongly expressed in the placenta and thought to have an accessory role in pathological angiogenesis, serving to potentiate the activity of VEGF (Carmeliet et al, 2001). Mice lacking the PlGFs gene are apparently otherwise normal (Persico et al, 1999). EG-VEGF is often

considered as a tissue selective angiogenic stimulator as its expression is largely restricted to steroidogenic glands, such as ovary, testis, adrenal cortex and placenta. Although it shows no structural homology with the other members of the VEGF family, it displays several striking similarities to VEGF by inducing endothelial proliferation and migration, by inducing fenestration in capillary endothelial cells derived from endocrine glands, and also for being regulated by hypoxia; explaining why it forms part of the VEGF family.

| VEGF receptor type | Ligands | Roles |
|---|---|---|
| **VEGFR-1** | VEGF, VEGF-B, PlGFs | VEGF signalling, release of growth factors. Vascular endothelium. |
| **VEGFR-2** | VEGF, VEGF-C, VEGF-D, VEGF-E | Proliferation, migration, survival, angiogenesis. Vascular endothelium. |
| **VEGFR-3** | VEGF-C, VEGF-D | Proliferation, migration, survival, angiogenesis. Mostly in lymphatic endothelium |

**Table 1.6 Roles of VEGF receptors and their receptor agonists.**

1.6.5.4 VEGF RECEPTORS

VEGF binding sites were first identified on the cell surface of vascular endothelial cells *in vitro* (Plouet and Moukadiri, 1990; Vaisman et al, 1990) and *in vivo* (Jakeman et al, 1992; 1993). Subsequently, VEGF receptors were found on bone marrow-derived cells such as monocytes (Shen et al, 1993). VEGF signals through two tyrosine kinase receptors, VEGFR-1 (previously termed Flt-1) and VEGFR-2 (previously Flk-1/KDR) which are expressed mainly on endothelial cells (see Table 1.6). Both VEGFR-1 and VEGFR-2 have seven immunoglobin–like domains in the extra-cellular domain, a single-transmembrane region, and a

consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Shibuya et al, 1990; Matthews et al, 1991; Terman et al, 1991). VEGFR-2 has been found to be responsible for signalling, whereas VEGFR-1 may play a regulatory role (Risau, 1997). A third VEGF receptor is VEGFR-3 (previously known as Flt-4), found in the lymphatic endothelium (Pajusola et al, 1992). It is however not a receptor for VEGF, but instead binds VEGF-C and VEGF-D (Karkkainen et al, 2002). It has also been shown that recombinant soluble VEGFR-1 or VEGFR-2 inhibits angiogenesis in the retina, corpus luteum, and tumours (Aiello et al, 1995; Ferrara et al, 1998; Lin et al, 1998), whereas expression of a dominant negative VEGFR-2 mutant using a retrovirus inhibits signalling through the receptor and suppresses tumour growth such as glioblastoma multiforme in vivo (Millauer et al, 1994; 1996). The expression of a soluble VEGFR-3 has been shown to inhibit lymphangiogenesis in mouse embryos (Makinen et al, 2001). In addition to these receptors, VEGF interacts with a family of co-receptors, the neuropilins (Soker et al, 1998) that seem to be involved primarily in modulating binding to the main receptors, although roles in signalling for these have not been ruled out.

### 1.6.5.5 BIOLOGICAL PROPERTIES OF VEGF

VEGF is a pleiotropic growth factor that mediates multiple functions through its stimulation of cognate receptors on endothelial cells (Figure 1.8).

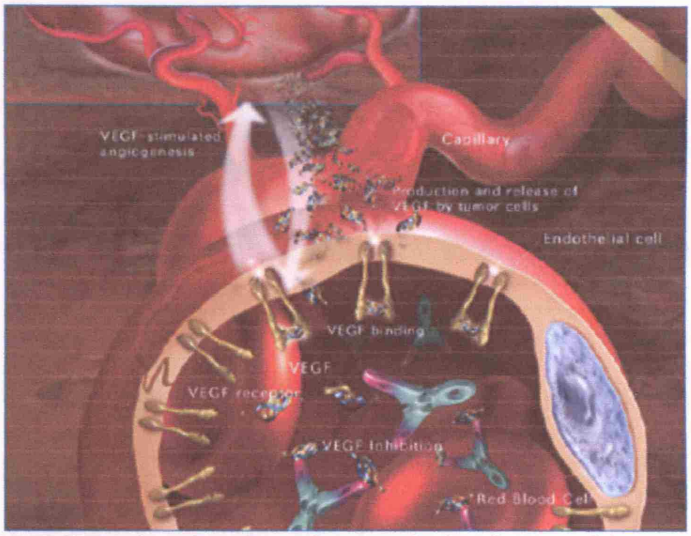



**Figure 1.8 VEGF-stimulated angiogenesis** (Ref: www.biooncology.com)

**1.6.5.5A Permeability**: In fact, it is one of the most potent inducers of vascular permeability known - 50,000 fold more potent than histamine (Dvorak, 2002). This ability to enhance vascular permeability is thought to be largely attributable to tumour cell expression, favouring tumour cell escape (Senger et al, 1983; Keck et al, 1989). It is suggested that the increase in microvascular permeability results in the leakage of several plasma proteins (fibrinogen and other clotting proteins) which can lead to the deposition of fibrin in the extravascular space, which as a result retards the clearance of oedema fluid and transforms the normally antiangiogenic stroma of normal tissues into a proangiogenic environment (Dvorak et al, 1995, Dvorak 2002). VEGF therefore increases permeability in a variety of vascular beds (skin, peritoneal wall, mesentery and diaphragm) and can lead to pathological conditions such as malignant ascites (Yoshiji et al, 2001) and malignant pleural effusions (Yuan et al, 1996). There is evidence that inhibition of VEGF can lead to a reduction in the formation of pleural effusions and that antibodies directed against VEGF or VEGFR-2 can also lead to a decrease in tumour vessel permeability and ascites formation (Yuan et al, 1996; Shaheen et al, 2001). The precise mechanisms by which VEGF increases permeability are

however not entirely clear. Recent work suggest that VEGF permeability may be induced either via a calcium-dependent pathway involving nitric oxide (Bates and Harper, 2002), or via an inter-endothelial cell pathway by opening junctions between adjacent endothelial cells (Ferrara, 2000; Zhang et al, 2002).

**1.6.5.5B Endothelial cell activation:** VEGF exerts a number of different effects on the vascular endothelium and endothelial cells. These effects include changes in endothelial cell morphology, cytoskeleton alterations and stimulation of endothelial cell migration and growth. It causes increased expression of several different endothelial cell genes (pro-coagulant factor, fibrinolytic pathway proteins, MMP, integrins, nitric oxide) and a variety of mitogens (Brooks et al, 1994; Zachary, 2001; Eliceiri and Cheresh; 1999; 2001; Dvorak, 2002). VEGF also induces vasodilation in vitro via the release of endothelial cell nitric oxide and prostaglandins and produces hypotension, transient tachycardia and decreases in cardiac output when injected into rats (Ferrara et al, 2003). This effect partly accounts for the hypertension and headaches occasionally observed in anti-VEGF clinical trials.

**1.6.5.5C Proliferation**: VEGF is a specific mitogen for several vascular endothelial cells derived from arteries, veins, and the lymphatic system (Keck et al, 1989; Ferrara and Henzel, 1989; Leung et al, 1989; Connolly et al, 1989; Plouet et al, 1989; Conn et al, 1990). In three-dimensional models, it acts as an angiogenic molecule by promoting confluent microvascular endothelial cells to invade collagen gels and to form capillary like structures (Pepper et al, 1992). However, *in vivo*, it is a specific growth factor causing sprouting of the

endothelium of rat aortic rings by proliferation of exclusively vascular endothelial cells (Nicosia et al, 1994).

**1.6.5.5D Invasion and migration:** Degradation of the basement membrane is necessary for endothelial cell migration and invasion, and is an important early step in initiating angiogenesis. VEGF induces a variety of enzymes (MMP, collagenase, serines proteases such as urokinase-type plasminogen activator) and proteins (tissue type-plasminogen activator) essential in the degradation process (Zachary and Gliki, 2001; Choong et al, 2003). It also promotes the expression of vascular cell adhesion molecules 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) in endothelial cells, enhancing the adhesion of Natural Killer cells and increases leukocyte adhesion (Melder et al, 1996). It is also known as a chemo-attractant for macrophages and promotes colony formation, attracting macrophages to the site of the tumour (Broxmeyer et al, 1996).

**1.6.5.5E Survival:** VEGF was first shown to act as a 'survival' factor for retinal endothelial cells (Alon et al, 1995) and has now been shown to promote survival in several endothelial cell in vitro and in vivo models (Ferrara et al, 2003). *In vitro*, it inhibits apoptosis in addition to upregulating anti-apoptotic proteins such as bcl-2; this, in turn, up-regulates members of the inhibitors of apoptosis family including survivin (Gerber et al, 1998; Zachary, 2001). *In vivo*, inhibition of VEGF results in extensive apoptotic changes in immature (without pericyte coverage) retinal cells (Alon et al, 1995) and in the developing vasculature of neonatal mouse but not that of adult mice (Gerber et al, 1999). Injection of exogeneous VEGF has shown to 'save' immature retinal vessels from destruction

(Alon et al, 1995) and VEGF dependence shown in endothelial cells of newly formed vessels but not that of established tumour vasculature (Benjamin, 1999).

**1.6.5.5F Other functions:** Professional antigen-presenting cells are very efficient at internalising antigen either by phagocytosis or by endocytosis. They usually display a fragment of the antigen, bound to a class II MHC molecule, on their membrane. VEGF has also been found to have an inhibitory effect on the maturation of host professional antigen-presenting cells such as dentritic cells resulting in silencing of the immune response of the body to the tumour (Gabrilovich et al, 1996) and to delay senescence (cell ageing) and induce escape from that process in human dermal micro-vascular endothelial cells - HDMEC (Arthur et al, 1998).

1.6.5.6 REGULATION OF VEGF GENE EXPRESSION

Although constitutively expressed by many tumour cells, VEGF gene expression is regulated by a number of control mechanisms including hypoxia (low oxygen levels), growth factors and cytokines (Ferrara, 2000).

**1.6.5.6A Oxygen concentration:** Hypoxia upregulates the expression of VEGF, but not of other VEGF family members, (Eriksson and Alitalo, 1999), by both increasing mRNA transcription and stabilisation (Claffey and Robinson, 1996; Shih and Claffey, 1998; Levy et al, 1995; Forsythe et al, 1996). Hypoxic transcriptional regulation of VEGF is mediated by hypoxia-inducible factor (HIF-1) binding, a heterodimeric protein transcription factor. HIF-1 alpha (HIF-1$\alpha$) is degraded rapidly under normoxic conditions by the ubiquitin pathway; however,

when stabilised by hypoxia, HIF-1α dimerises with HIF-1β, and the complex binds to and activates the VEGF promoter. Hypoxia induced-VEGF over-expression is regularly associated with the up-regulation of VEGFR-1 and VEGFR-2. It has also been reported that hypoxia upregulates VEGF receptors expression independent of VEGF (Gerber et al, 1997). The importance of hypoxic regulation of VEGF expression for tumour malignancy and tumour angiogenesis is currently not fully understood, because many tumours have shown an overexpression of VEGF even under normoxic conditions (Brown et al, 1997).

**1.6.5.6B Oncogenes and tumour suppressor genes:** As previously mentioned in sections 1.5.2 and 1.5.3, there is good evidence that oncogenes and tumour suppressor genes promote tumour growth, at least in part by modulating the angiogenic response induced by VEGF. Oncogenes (*SRC*, *RAS*) and the *P53* and Von Hippel Lindau (*VHL*) tumour suppressor genes all play crucial roles in the regulation of VEGF expression in different human tumours (Mukhopadhyay et al, 1995; Rak et al, 1995a, b; 2001; Ohh and Kaelin, 1999). The *VHL* tumour suppressor gene product forms part of a protein complex that targets specific proteins, including HIF-1α for ubiquitinylation and proteolysis. Therefore, when *vHL* is absent or inactivated, as commonly occurs in renal cell carcinoma, HIF-1α and thus the HIF complex is stabilised even under normoxic conditions with result in up-regulation of VEGF and perhaps other members of the VEGF family (Ohh and Kaelin, 1999; Clifford and Maher, 2001; Pal et al, 1997; Siemeister et al, 1997). The *P53* tumour suppressor gene plays a central regulatory role of VEGF in malignant tumours by suppressing VEGF transcription, for example, in breast cancer cells (Pal et al, 2001).

**1.6.5.6C Cytokines and hormones:** Inflammatory cytokines such as IL-1-α and IL-6 have been found to induce the expression of VEGF in several cell types, including synovial fibroblasts, in agreement with the hypothesis that VEGF may be a mediator of angiogenesis/ permeability in inflammatory disorders (Ben-Av et al, 1995; Cohen et al, 1996). Cells that make steroid hormones (adrenal cortex, corpus luteum, Leydig cells) have also been shown to express VEGF and these cells themselves are under hormonal regulation – for example, the cycling uterus and ovary (Berse et al, 1992; Cullinan-Bove et al, 1993; Garrido et al, 1993). Peptide hormone-producing cells also express VEGF, including thyroid follicular cells and its production in culture is up-regulated by a number of agents including insulin (Sato et al, 1995). In addition, thyroid stimulating hormone up-regulates VEGF mRNA expression in human thyroid follicle and promotes VEGF secretion in several thyroid cancer cell lines (Soh et al, 1996).

**1.6.5.6D Growth factors:** Several major growth factors including EGF, PDGF, IGF-I, TNF-α, TGF-α/β, upregulate VEGF mRNA expression (Frank et al, 1995; Pertovaara et al, 1994; Warren et al, 1996) suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in the regulation of VEGF release in the microenvironment. Recently, IGF-I has been shown to induce angiogenesis by increasing VEGF production in pancreatic carcinomas (Zeng et al, 2003); colorectal carcinomas (Warren et al, 1996) and endometrial adenocarcinomas (Bermont et al, 2000). Only *in vitro* studies have shown the importance of IGF-I, among many growth factors, in the local regulation of ovarian function (Adashi, 1995; Giudice, 1992; Gougeon, 1996) while IGF-1 receptor (IGF-1R) is often over-expressed in a variety of human cancers (breast,

endometrial, pancreatic and colorectal), and has been associated with aggressive disease and formation of metastases (Reinmuth et al, 2002).

1.6.5.7 ANTI-VEGF THERAPY

Anti-VEGF therapy has been used in clinical trials to inhibit angiogenesis and is showing promising results in cancer treatment therapies. Bevacizumab, a recombinant humanized monoclocal antibody to VEGF that recognises all isoforms of VEGF, is the first of a new class of cancer drugs. It has been shown to induce apoptosis of breast cancer cells and is currently used in combination with intraveinous 5-FU for the first-line treatment of metastatic colorectal cancer (Wedam et al, 2006). Figure 1.9 demonstrates the action of Bevacizumab on a tumour.

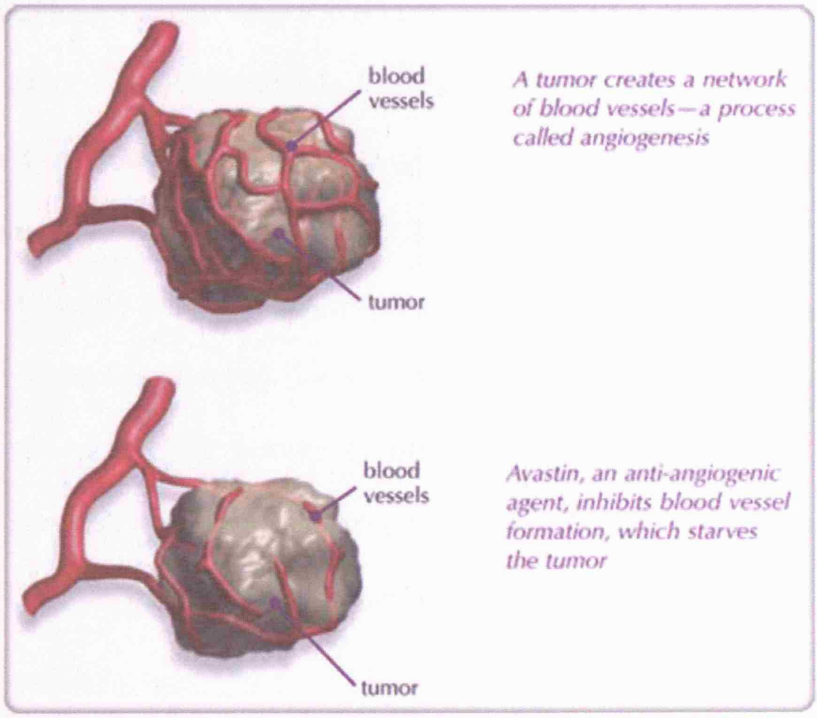



**Figure 1.9 Bevacizumab (Avastin; Genentech Inc, CA, USA)**
(Ref: www.avastin-info.com)

There are other agents that target VEGF receptors and are currently being used in clinical trials. They are VEGFR antibodies, small-molecule kinase inhibitors and ribozymes. Neutralizing anti-VEGFR-1 antibodies have been shown to inhibit receptor signalling and migration in response to VEGF stimulation (Kanno et al, 2000; Lu et al, 2001). Small molecule kinase inhibitors are low-molecular weight, ATP-mimetic proteins that bind to the ATP-binding catalytic site of the tyrosine kinase domain of VEGFRs, resulting in a blockade of intracellular signalling (Gourley et al, 2000; Sun et al, 2000). Ribozymes are catalytic RNA molecules that can splice or cleave other RNA molecules, rendering the mRNA untranslatable and therefore down-regulates the expression of any endogenous gene products (Sandberg et al, 1999).

### 1.6.6 PLATELET DERIVED ENDOTHELIAL CELL GROWTH FACTOR / THYMIDINE PHOSPHORYLASE (PD-ECGF/TP)

The fact that PD-ECGF/TP is an angiogenic enzyme involved in nucleotide homeostasis and not a classic growth factor makes it a particularly intriguing molecule to study. It is now known that a by-product of thymidine phosphorylase action on thymidine, 2-deoxy-D-ribose, is angiogenic and it is release of this that mediates the angiogenic activity of the enzyme. Gliostatin, which stimulates neuronal survival, has also been shown to be PD-ECGF/TP (Asai et al, 1992). In this thesis, PD-ECGF/TP is referred to simply as TP.

#### 1.6.6.1 ISOLATION AND GENE STRUCTURE

The enzyme TP was first purified and characterised as an enzyme involved in nucleic acid homeostasis; a role that was well established long before its

angiogenic activity. It was first described in 1954 (Friedkin and Roberts, 1954) and purified in the mid-1970s from both *Escherichia coli* and *Salmonella typhimurium* (Blank and Hoffee, 1975; Voytek, 1975). Bacterial TP is a homodimer of 45 kD (Walter et al, 1990). Eukaryotic TP, a 47 kD subunit homodimer, was first purified from the human amniochorion (Kubilus et al, 1978; Gan et al, 1981).

In 1987, a molecule extracted from human platelet lysates was found to stimulate proliferation of aortic endothelial cells in vitro and became known as PD-ECGF, not to be confused with platelet-derived growth factor – PDGF, (Miyazono et al, 1987). In 1989, PD-ECGF was purified (Miyazono and Heldin, 1989) and PD-ECGF gene isolated (Hagiwara et al, 1991). The gene was localised on chromosome 22q13.33 and is composed of 10 exons dispersed over a 4.3 Kb region. Its promoter lacks a TATA box and a CCAAT box, structures characteristic of eukaryotic promoters (Hagiwara et al, 1991). In 1992, a protein sequence search revealed that PD-ECGF showed significant sequence identity with *E.coli* TP (Usuki et al, 1992). Later PD-ECGF was recorded to have similar effects to those of TP, showing angiogenic properties by stimulation of endothelial cell migration (Moghaddam et al, 1995). Subsequently, PD-ECGF and TP were confirmed as the same molecule (Moghaddam et al, 1995).

1.6.6.2 BIOLOGICAL ACTIVITIES

**1.6.6.2A Catalysis**: TP possesses enzymatic functions to catalyse the reversible phosphorylitic breakdown of thymidine to 2'-deoxyribose 1'- phosphate and thymine (Zimmerman et al, 1964; Gallo and Perry, 1969). In turn, 2'-deoxyribose 1'- phosphate is rapidly de-phosphorylated to 2'-deoxy-D-ribose, which is

transported out of the cell (Usuki et al, 1992) and shown to be angiogenic in the chick chorioallantoic membrane (CAM) assay (Haraguchi et al, 1994; Moghaddam et al, 1995), in which embryologists introduced tissue grafts into an egg shell and placed onto the CAM to study the developmental potential of embryonic tissue grafts. Although the enzyme reaction is reversible, the role of TP is thought to be primarily catabolic, regulating thymidine levels in cells, since thymidine is toxic to cells at high concentrations and causes an alteration in DNA replication and repair. However, high levels of TP have been found in macrophages, suggesting other functions in these cells such as angiogenic properties. Hypoxia has been demonstrated to affect the regulation of TP *in vitro* as well as *in vivo* (Griffiths et al, 1997).

**1.6.6.2B Angiogenesis:** TP possesses an angiogenic activity that has been confirmed in a number of *in vivo* assays. These include the CAM assay, the rabbit corneal assay, the rat subcutaneous assay, the injured skin graft assay, the gelatin implant assay, and in xenografted PD-ECGF/TP transfected cell lines (Taylor and Weiss, 1985; Ishikawa et al, 1989; Lees and Fan, 1994; Miyadera et al, 1995; Moghaddam et al, 1995). In most of these assays, introduction of TP, either by injection, incubation, exposure or implantation, leads to an enhancement of neovascularisation in each of the assays. The fact that angiogenic activity was seen in such a varied range of established assays was reasonable to conclude that TP is clearly angiogenic. In addition it was found that its angiogenic activity depends upon its enzymatic activity, as mutagenesis of the enzyme's active–site residues abolished the angiogenic activity (Moghaddam et al, 1995; Miyadera et al, 1995).

**1.6.6.2C Cell trophism:** TP has the ability to promote chemotaxis and cell growth in endothelial cells in vivo and angiogenesis in vitro (Ishikawa et al, 1989). The Boyden chamber – commonly used to analyse chemotactic activity of molecules– was used by Ishikawa *et al* (Ishikawa et al, 1989) to demonstrate that TP is chemotactic for bovine aortic endothelial cells (Ishikawa et al, 1989). This activity was subsequently confirmed *in vitro* by Miyadera *et al.* and Moghaddam *et al.* (Miyadera et al, 1995; Moghaddam et al, 1995). As TP is not a mitogen for endothelial cells *in vitro*, induction of migration of endothelial cells is likely to be part of the mechanism by which this enzyme stimulates angiogenesis *in vitro*. Recruitment of inflammatory cells by TP is also likely to contribute to its angiogenic activity, as a chemotaxis response by neutrophils and monocytes to TP (Miyazono et al, 1991).

**1.6.6.2D Tumour growth:** There is strong evidence that TP enhances tumour growth. Ishikawa *et al.* transfected the cDNA for TP into a *RAS*-transformed NIH 3T3 cell line, al-1, in nude mice, and found a dramatic increase of vascular density in tumour cells over-expressing TP (Ishikawa et al, 1989). TP has also been transfected into MCF-7 cells that have the potentiality of forming slow growing tumours. Histological examination of these tumours after transfection revealed that the tumour mass was carcinoma and not mouse-derived inflammatory cells infiltrating and enlarging the tumour.

**1.6.6.2E Apoptosis:** TP has been shown to act as an anti-apoptosis factor. It is associated with the anti-apoptosis mechanism, especially in cells damaged by stress, caused by factors such as hypoxia (Kitazono et al, 1998; Ikeda et al, 2003)

or chemotherapeutic drugs (Ikeda et al, 2003). Promotion of angiogenesis and inhibition of apoptosis by TP may explain why tumours that over-express TP have unfavourable prognosis (Toi et al, 2005).

1.6.6.3 REGULATION OF TP

**1.6.6.3A Inflammatory mediators and cytokines:** Cancer cells contain large amounts of inflammatory mediators and are associated with physical stress. In the early stages of certain (non invasive or mucosal) cancers, cytokine-rich conditions and hypoxic microenvironments take place. The up-regulation of such mediators and the involvement of stress cause additional changes in the genomic or proteomic regulation in tumour cells, and affect the phenotype of neighbouring stromal cells. Cytokines such as TNF-$\alpha$, IL-1 and IFN $\gamma$ can up-regulate TP expression in malignant cells (Eda et al, 1993; Goto et al, 2001; Fukushima et al, 2002). Hypoxia or hypoglycaemic conditions has also been shown to stimulate TP expression (Griffiths et al, 1997), indicating that TP is a product of inflammation or microenvironmental stress.

**1.6.6.3B Transcriptional control:** In pathological conditions, the transcriptional step of TP is still unclear. The human epidermoid carcinoma cell line (A431) was reported to express 3.0 and 3.2 kb transcripts of TP in addition to the originally identified 1.8 kb transcript (Usuki et al, 1994). In these additional transcripts, there were seven of eight copies of binding sites of the transcription factor (SP1) that are present in the transcription promoter region of the 1.8 kb transcript, important in inducing TP expression (Zhu et al, 2002).

**1.6.6.3C Anti-tumour treatments:** TP is unique related to cancer treatment because *in vitro* studies have shown that cancer cells pre-treated with IFN-α induce TP that metabolises a number of thymidine analogues, including 5'-deoxy-5-fluorouridine (Eda et al, 1993). This compound is of therapeutic interest because it is converted to 5-fluorouracil (5FU) and its derivatives, by TP. The ability of 5-FU to slow and eventually inhibit DNA synthesis by interfering with the activity of an enzyme (thymidylate synthetase, TS) has made it one of the most successful and widely used chemotherapeutics for the treatment of solid tumours, particularly for colorectal cancer (Longley et al, 2003). Its derivatives such as doxifluridine and capecitabine (Sawada et al, 1998; Endo et al, 1999) also inhibit tumour growth and are especially specific because conversion of these drugs to their active form depends on TP. Findings from clinical trials have also suggested that capecitabidine in combination with other cytotoxic agents, provide favourable effects for response rates, time to progression and survival for patients with metastatic cancer compared with the anti-metabolite alone (Cassidy et al, 2004).

## 1.6.7 OTHER STIMULATORY AND INHIBITORY AGENTS

There are other growth factors, chemicals, proteins, and processes that are important promoters or inhibitors in tumour growth. Stimulatory angiogenic factors, including Angiopoietin (Ang) 1/2, work in collaboration to promote angiogenesis (Holash et al, 1999) whereby Ang-1 functions as an agonist whereas Ang-2 acts as antagonist at this receptor (Maisonpierre et al, 1997).

Clinically, patients have been treated with direct angiogenesis inhibitors, such as IFN-α, since 1997 and endostatin since 2001. At present, a wide range of clinical trials are still under way to determine the efficacy and toxicology associated with

anti-angiogenic strategies such as those involving angiostatin, endostatin, 2-methoxyestradiol and vitaxin. This approach is of low toxicity without any of the side effects associated with chemotherapy and there is no drug resistance (Folkman, 1993). Furthermore, several studies have indicated that combining direct angiogenesis inhibitors with cytotoxic therapy results in a full and sustained regression of large established tumours without overt toxicity (Klement et al, 2000; Takahashi et al, 2001). Anti-angiogenic gene therapy is also being tested in animal models (Folkman, 1998) to assess the inhibition of angiogenic growth factor expression that may be used with antisense mRNAs or ribozymes administered in viral or liposomal vectors.

### 1.6.8 MICROVESSEL DENSITY (MVD)

The degree of angiogenesis of a tumour, as assessed by MVD, has emerged as a powerful candidate for prognosis and as a predictive tool (Weidner, 1998). In a multivariate analysis, MVD was found to be the most accurate prognostic indicator in breast carcinoma for disease-free survival (DFS), better than size, grade, or oestrogen receptor status (Jacquemier et al, 1998). In other studies, VEGF expression has correlated with increased MVD and poor prognosis in several tumour types (Takahashi et al, 1995; Ogawa et al, 1995). Many studies have used measurements of MVD in regions of high vessel density (HVD), also termed as 'hotspots', to assess the influence of tumour angiogenesis on prognosis. Early studies (Srivastava et al, 1988; Bosari et al, 1992) showed a prognostic value of MVD in breast cancer and cutaneous melanoma. Further studies have shown a link between MVD and prognosis in several solid tumours, such as those of the breast, lung, prostate, ovary, head and neck, cervix, oesophagus, colon, and

non-small cell lung carcinomas (Macchiarini et al, 1992; Takahashi et al, 1995; Tae et al, 2000). Kato *et al.* confirmed the prognostic usefulness of tumour MVD in 377 Japanese patients with breast cancer, followed for a median of 10 years (Kato et al, 2001). A raised MVD was associated with both low-relapse free and overall survival.

### 1.6.8.1 MARKERS OF BLOOD VESSELS

A variety of endothelial cell markers have been used to highlight tumour blood vessels immunohistochemically. The most commonly used antibodies include those against CD31/platelet-endothelial cell adhesion molecule (PECAM-1), CD34 and factor VIII-related antigen. CD31/PECAM-1 is a transmembrane glycoprotein involved in cell adhesion (DeLisser et al, 1994) and CD34 is a surface glycoprotein of unknown function (Krause et al, 1996). Both anti CD31/PECAM-1 and anti-CD34 have their own specific limitations. Anti CD31/PECAM-1 cross-reacts with plasma cells, fibroblasts and tumour cells; in those tumours with a high plasma cell infiltrate, it may even obscure the microvessels (Weidner N, 1993). Anti-CD34 recognises a cell surface antigen selectively expressed on haemopoietic progenitor cells and on some vascular cells, however, it also cross-reacts with perivascular stromal cells and fibroblasts (Traweek et al, 1991). Factor VIII-related antigen forms part of the von Willebrand factor (vWF) complex and plays a role in the coagulation process (Fay, 1993). Compared to the previous endothelial markers, anti-vWF provided a good contrast between microvessels and other tissue components (Weidner et al, 1992). Also, it is more sensitive in distinguishing benign reactive endothelial cells (Abulafia et al, 1999). At present, endoglin (CD-105) is also a sensitive

microvessel marker, capable of binding preferentially to the activated endothelial cells in angiogenesis (Westphal et al, 1993; Seon et al, 1997) and thus it is potentially a more specific marker for tumour neovascularisation. Further attempts to distinguish newly formed immature vessels from those that are more established and mature have also been carried out during the last decade. They are based on the use of antibodies to α-smooth muscle actin (α-SMA), which appear to stain mature vessels as they attract a 'coat' of peri-endothelial support cells – that is, smooth muscle (α-SMA)- positive cells (Benjamin et al, 1999).

### 1.6.9 INSULIN-LIKE GROWTH FACTORS

The insulin like growth factor (IGF) system plays an important role in normal and tumour cell biology (Daughaday, 1990; Cullen et al, 1991; LeRoith et al, 1995). IGF belongs to a family of polypeptides with considerable structural similarities to insulin and possess both anabolic and mitogenic properties both in vivo and in vitro (Blundell et al, 1983; Rinderknecht and Humble, 1978a; b). The IGF system is comprised of the ligands: IGF-I, IGF-II and insulin, 6 binding proteins (IGFBP), and cell surface receptors that mediate the actions of these ligands (IGF-IR, insulin receptor and the IGF-II mannose-6-phosphate [M-6-P] receptor). The work in this thesis focuses on IGF-I.

#### 1.6.9.1 IGF-I GENE STRUCTURE

In both humans and rats, the IGF-I gene consists of more than 70 kilobases and comprises 6 exons and at least 5 introns, and is located on chromosome 12q22. (Rotwein et al, 1986; Shimatsu and Rotwein, 1987; Rotwein, 1991). Two promoters, one adjacent to exon 1 and the other to exon 2 control gene

transcription. Both promoters lack a "TATAA" box, a "CCAAT" box and other typical proximal control elements, yet neither is similar to that of a GC-rich "housekeeping" promoter (Adamo et al, 1991; Jansen et al, 1991; Hall et al. 1992). Exons 1 and 2 are alternative leader exons derived from different transcription start sites, and encode part of the signal peptide (Jansen et al, 1983; Tobin et al, 1990). The resulting variant mRNA transcripts with different 5' untranslated regions (UTR) have been classified as Type/Class 1 (exon 1-3) and Type/Class 2 (exon 2-3). These 5'UTR mRNA variants are differentially regulated during development in a tissue specific manner (Lowe et al, 1988). Class 1 mRNA transcripts are expressed in the rat liver and a wide range of non-hepatic tissues both in prenatal and postnatal animals (Hoyt et al, 1988; Adamo et al, 1989). In contrast, Class 2 mRNA transcripts were barely present or absent in non-hepatic tissues (Hoyt et al, 1988; Adamo et al, 1991), suggesting that the basal promoter associated with exon 1 is active in a wide range of tissues.

In addition to transcription from two promoters, IGF-I is regulated by post-transcriptional events, which yield several mature mRNA transcripts. In all of these variants exons 3 and 4, which encode the mature 70 amino acid peptide are constant (Bell et al, 1986; Gilmour, 1994) whereas, exons 5 and 6 are subject to a complex alternative splicing pattern. This is a complex mechanism by which exons are arranged in different combinations from pre-mRNA. It is an important and common process for generating protein diversity and regulating gene expression in higher eukaryotes.

1.6.9.2 IGF-I PRIMARY STRUCTURE

The primary structure of IGF-I resembles pro-insulin as it consists of an amino terminal B region and an A region that is separated by a short connecting C domain. Unlike pro-insulin however it also has a D region extension peptide and an E peptide at its carboxyl terminus. The different E peptides are cleaved from the pre-pro-IGF-I to produce a single mature IGF-I peptide consisting of the B, C, A, and D domains (Kim et al, 1991; Jansen et al, 1991).

1.6.9.3 IGF-I mRNAS THAT ENCODE DIFFERENT E PEPTIDES

Alternative splicing of exons 5 and 6 of the IGF-I gene results in IGF-I mRNAs encoding different E peptides. Exon 4 usually splices to exon 6 (Jansen et al. 1983), but can also splice to exon 5, representing 1-10% of IGF-I transcripts (Rotwein, 1986). A schematic representation of the human IGF-I gene and the splicing pattern of its splice variants is shown in Figure 1.5. 3' alternative splicing of the primary IGF-I gene generates six different IGF-I mRNAs in humans: Class 1 or 2 IGF-IEa, IGF-IEb and IGF-IEc, and these are shown in Figure 1.10.

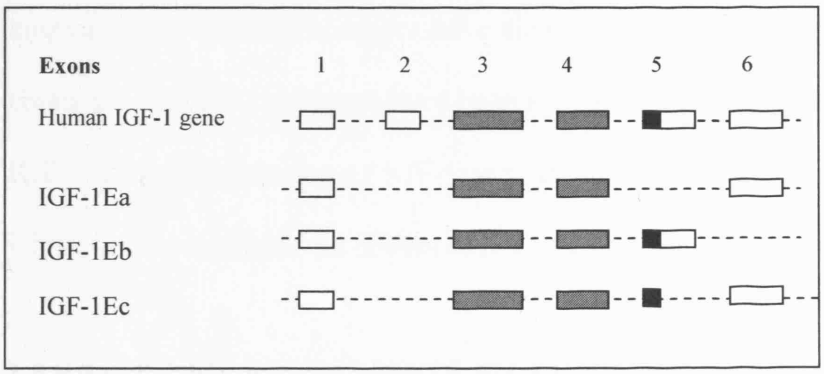



**Figure 1.10 Schematic representations of IGF-1 gene (class 1) and its variants.**

### 1.6.9.4 CELLULAR ACTIVITIES OF IGF-1

IGF-1 exerts acute anabolic actions on protein and carbohydrate metabolism by increasing the cellular uptake of amino acid and glucose and by stimulating glycogen protein synthesis (Jones and Clemmons, 1995). IGF-1 has also both immediate and long-term effects on various cellular activities such as cell proliferation, cell differentiation and apoptosis (Jones and Clemmons, 1995). IGF-1 is a potent mitogen for a wide variety of cells including fibroblasts, smooth muscle cells, epithelial cells, and exerts its mitogenic action by increasing DNA synthesis (Dufourny et al, 1997; Furlanetto et al, 1994).

### 1.6.9.5 IGF-1 AND CANCER

The possible involvement of IGF-1 in cancer was initially observed in cell culture experiments (Macaulay, 1992). Animal experiments in mice then followed demonstrating that the overexpression of IGF-1 increases the likelihood of tumour development in certain tissues (Rogler et al, 1994). At present, many molecules including estrogens and tumour suppressor genes such as PTEN and p53, that are known to be involved in cancer have also been found to interact with IGF-1. In breast cancer cells, estrogens have been shown to enhance the mitogenic effect of IGF-1, induce expression of IGF-1 and stimulate production of receptor IGF-1R, which in turn mediates the effects of IGF-1 on cancer cells (Clarke et al, 1997).

### 1.6.9.6 IGF-1 SPLICE VARIANTS AND MUSCLE REPAIR

Studies performed in rats have demonstrated that muscle tissue was capable of expressing IGF-1Ec locally in response to mechanical signals and / or damage (Goldspink, 1999; 2002; Goldspink et al, 1996). The evidence indicates that the two splice variants have different roles with the role of IGF-1Ec after muscle

injury to activate muscle satellite cells proliferation. Satellite cells, in normal undamaged tissue, are quiescent and usually detected just beneath the basal lamina. They express M-cadherin and when activated, they start coexpressing myogenic factors and later myogenin. The initial activation of muscle satellite cells is important because muscle is a post-mitotic tissue and the activated satellite cells fuse with muscle fibres to provide the extra nuclei for muscle fibre repair and hypertrophy. Later the IGF-1 gene is spliced to the isoform IGF-1Ea, which recent studies suggest provide the main anabolic response that is involved in generally up-regulating protein synthesis (Hill and Goldspink, 2003).

## 1.7 LYMPHANGIOGENESIS

Lymphangiogenesis is the growth of newly formed lymphatic vessels (Jackson et al, 2001; Karkkainen et al., 2001, 2002; Pepper, 2001; Sleeman et al., 2001). These vessels are part of the circulatory system. The lymphatic system is made up of an extensive network of capillaries, collecting vessels and ducts that permeate most organs (Ryan et al, 1986). Unlike the blood vasculature that forms a continuous loop, the lymphatic system is a one-way, open-ended transit system. Under physiological conditions, these vessels collect the extravasated protein-rich lymph and lymphocytes from the tissues. From the lymphatic capillaries, the fluid is transferred to the collecting lymphatic vessels and ultimately into the venous circulation via the thoracic duct. Larger lymphatic vessels are surrounded by a muscular layer that contracts automatically when the vessel becomes stretched with fluid. In addition, external factors such as skeletal muscle contractions or arterial pulsations compress the vessels and increase the efficiency of fluid transport.

### 1.7.1 LYMPHATIC AND BLOOD VESSELS

In tissue sections, the lymphatic vessels differ in many ways from the blood vessels, but they also have similar properties (Baldwin et al, 2002). Both vessel types are lined by endothelium but lymphatics generally have thinner and irregular walls. Blood vessels have a continuous or fenestrated basement membrane and tight inter-endothelial junctions, making the vessel wall selectively permeable to cells, fluids, and molecules, whereas lymphatic vessels have complex overlapping intercellular junctions holding the vessel open as tissue pressure rises, thus permitting free import of interstitial fluid (Witte et al, 1997). Extravasated fluid and solutes from the extracellular spaces are collected by the initial lymphatics, and then passed through a network of progressively larger lymphatic vessels, finally returning to the blood circulation via the thoracic duct (Casley-Smith, 1980). Lymph contains few red blood cells and platelets and is therefore much less coagulable than blood. It is drained from the peripheral tissues and consists of interstitial tissue fluid, and metabolites (Witte et al, 1997).

In 1627, Asellius was the first to characterise the lymphatic system (Asellius, 1627), at about the same time that the blood circulation was described by William Harvey (Harvey, 1628). However, compared with blood vascular studies, studies of the lymphatic system have remained relatively neglected until recently. One of the major limitations of research on lymphatic vessels and lymphangiogenesis was due to the lack of histological, ultrastructural, and immunohistochemical markers to accurately differentiate blood endothelial cells from lymphatic endothelial cells (Sauter et al, 1998; Clarijs et al, 2001; Jackson, 2001; Partanen and Paavonen, 2001; Pepper, 2001; Sleeman et al, 2001). In the past few years, however, several markers specific for lymphatic endothelium have been discovered such as

VEGFR-3, lymphatic vessel endothelial-receptor-1 (LYVE-1, Banerji et al, 1999) and PROX-1 (Wigle and Oliver, 1999). Among these, VEGFR-3 is predominantly expressed by lymphatic endothelial cells in normal adult tissues, but can also be expressed by blood vascular endothelial cells in tumours or during wound healing. To date, two lymphangiogenic factors, which are members of the VEGF family, have been identified. They are VEGF-C and VEGF-D, as demonstrated in a number of experimental systems including the CAM (Oh et al, 1997), and the rabbit cornea assay (Szuba et al, 2002); these systems have the dual capacity to induce lymphangiogenesis and angiogenesis.

### 1.7.2 LYMPHANGIOGENESIS IN TUMOURS

In tumours, it was long thought that lymphatic vessels were an inactive part of the stroma (Tanigawa et al, 1981; Jain, 1987; Baxter and Jain, 1990; Folkman, 1996; Leu et al, 2000; Karkkainen et al, 2002). However, in 2001, several studies in experimental xenotransplanted tumour models elegantly demonstrated that tumour lymphatic vessels do exist, and that they are largely dependent on the activation of VEGFR-3 by either VEGF-C or VEGF-D (Karpanen et al, 2001; Mandriota et al, 2001; Skobe et al, 2001a,b; Stacker et al, 2001).

Metastatic spread is a major hallmark of almost all malignant neoplasms and is the major cause of cancer death (Hanahan and Weinberg, 2000; Weiss, 2000). There are 4 main routes of tumour spread: (1) local invasion; (2) direct seeding to body cavities (peritoneum, pleura, pericardium); (3) haematogeneous spread; (4) lymphatic spread. Epithelial malignant tumours preferentially disseminate to regional lymph nodes initially and then to distant sites (Sleeman, 2000; Weiss, 2000; Pepper, 2001). There are 3 main reasons, which make the lymphatic system

a preferential pathway for metastatic dissemination. Firstly, lymphatics are much larger than blood capillaries and do not possess a continuous basement membrane. Secondly, inside the lymphatic system, the flow velocities are much lower than those observed in the blood system. Thirdly, the lymph composition is much the same as the interstitial fluid and is able to promote cell viability (Pepper, 2001; Sleeman et al, 2001; Swartz, 2001; Swartz and Skobe, 2001). In contrast, the blood stream is a highly aggressive medium for neoplastic cells owing to high shear stresses and mechanical deformation (Weiss, 1992; Swartz, 2001; Swartz and Skobe, 2001). Also, metastasis is known to be a low-efficiency event and a significant number of the neoplastic cells are dormant or in apoptosis in the blood (Mehes et al, 2001; Naumov et al, 2001).

## 1.8 OVARIAN ANGIOGENESIS

The tissues of the human female reproductive tract are unique regarding the constant specific changes in vascularisation that occur each month throughout reproductive life. The female reproductive system is therefore a good model for the study of the regulation of angiogenesis.

### 1.8.1 IN THE NORMAL OVARY

Under physiological conditions, a number of changes take place in the vascularisation of the ovary on a cyclical basis. Just before or after birth, the ovary of most mammals contains a high number of non-growing, primordial follicles, each of which is composed of an oocyte surrounded by a layer of squamous pre-granulosa cells (Greenwald and Terranova, 1988). These primordial follicles strongly depend on the surrounding stromal vessels for delivery of nutrients and

hormones. With time, the primary follicles develop an initial vascular supply until a rich vascular supply is set up. Usually, the selection of the dominant follicle during each menstrual cycle depends on the formation of a rich vascular supply and increased follicular permeability. Thus the exact regulation of ovarian angiogenesis during the follicular phase of the menstrual cycle is crucial to the events leading up to ovulation. At ovulation, vascular development of the dominant follicle becomes more intensive, with the development of the corpus luteum requiring an essential source of lipids e.g. cholesterol needed for the synthesis of progesterone (Clark 1900; Andersen 1926; Bassett 1943). About 50% of the cells of the mature corpus luteum are endothelial cells and the majority of parenchymal (steroidogenic) cells are adjacent to one or more capillaries (Dharmarjaran et al, 1985; Niswender and Nett, 1988). Most of the ovarian blood supply reaches the mature corpus luteum and this vascularisation has shown a high correlation with progesterone secretion (Reynolds, 1986; Niswender and Nett, 1988). In situations when any alteration happens in the functioning of the corpus luteum, as a result of, for example, insufficient vascularisation, the luteal progesterone production is altered (i.e. luteal phase defect) and eventually this results in regression (Reynolds 1986; Niswender and Nett, 1988), suggesting the coordinated action of stimulators and inhibitors of angiogenesis (Goede et al, 1998). Studies have shown that VEGF mRNA expression is temporally associated with the proliferation of blood vessels in the ovary (Phillips et al, 1990).

### 1.8.2 IN EOC

In the last decade, many studies have attempted to unravel the role played by angiogenesis in benign and malignant neoplasms of the ovary. Despite the

existence of established prognostic markers for EOC, there is still a lack of clinically reliable molecular markers for assessing diagnosis. It remains unclear whether benign ovarian neoplasms represent a precursor of ovarian cancer, analogous to the adenoma-carcinoma sequence in colorectal carcinoma, or whether these are separate entities. To my knowledge, this study is the first to analyse a combination of several markers such as VEGF, VEGF-C, TP, IGF-1 and MVD in normal, benign and malignant neoplasms of the ovary.

## 1.9 HYPOTHESIS

It is hypothesised that benign ovarian neoplasms and EOC may have common angiogenic pathways; with a progression from benign ovarian neoplasms to ovarian carcinomas involving similar patterns of expression of the angiogenic proteins. As a result, the combination of several angiogenic and lymphangiogenic factors examined in EOC may reveal such specific markers valuable in identifying those cases which could progress to malignancy.

The hypothesis which will be examined in this thesis will attempt to answer the following questions:

1. Are there molecular differences, at the tissue and fluid levels, and protein and mRNA levels of these angiogenic markers between EOC and potentially pre-malignant ovarian neoplasms?
2. Can such molecular markers be of prognostic value for potentially pre-malignant lesions and do they correlate with other parameters in EOC such as histological subtype, tumour stage and disease grade?
3. Could the serum levels be useful to determine the progression of potentially premalignant ovarian neoplasms to EOC?

## 1.10 AIMS OF THIS RESEARCH PROJECT

The aims of this project are therefore:

- to identify prognostic markers in potentially premalignant lesions of the ovary and EOC, using molecular and morphological techniques,
- to understand the molecular pathways of angiogenesis and lymphangiogenesis in EOC.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 REAGENTS

All laboratory reagents are listed along with the manufacturer in Appendix I. The grade of reagents, where applicable, is also given. Molecular biology grade reagents were used in methods involving PCR, e.g. RNA extractions, PCR, quantitative real-time RT-PCR and gel electrophoresis.

### 2.1.2 CLINICAL SAMPLES

The study was conducted at the Royal Free Hospital and ethical approval from the Local Research Ethics Committee (LREC) was obtained for tissue and fluid collection.

Tissue was removed during routine surgery and either snap-frozen or placed in formalin and processed routinely through the Department of Histopathology, where the tissue was paraffin wax-embedded. Tissue fluids (serum, cystic and ascitic fluids) were obtained prior to or during surgery and immediately snap frozen and stored at -80°C.

Depending on the availability of the samples, the number examined differed for each part of this study. These samples are described in the respective chapters of the thesis.

#### 2.1.2.1 FROZEN TISSUE

Once a fresh biological sample is obtained from surgery, its RNA becomes very unstable during handling and processing of the sample, resulting in the enzymatic degradation of RNA. Immediate stabilisation of the RNA expression pattern is therefore a prerequisite for accurate gene expression analysis. As a result, all

tissues obtained from surgical procedures were either immediately submerged into liquid nitrogen or immersed immediately into a stabilisation reagent, such as RNAlater™ RNA stabilisation reagent, and then stored at -80°C, for maximum protection of the RNA. When required, these frozen tissues were placed on dry ice as thawing must be avoided to prevent any RNA degradation and alteration of the RNA profile. A small portion of the 'frozen' tissue, approximately 20-30 mg, was first placed on dry-ice, then cut and weighed under cold conditions (in a cold room at 4°C) to avoid rapid thawing. This tissue was then immediately used for RNA extraction.

2.1.2.2 ARCHIVAL TISSUE

There are a number of methods for fixing tissue to preserve its morphology, depending on the tissue type and which techniques will be used after sectioning. The most widely used fixatives in diagnostic hospital histology laboratories are formalin based, as formalin is a neutral salt employed to maintain tonicity. In this study, either 10% formol saline or 10% neutral buffered formalin (10% w/v formaldehyde in water) was used. After fixation was complete, the fixative was poured off and the tissue was processed using an enclosed automatic processing system (VIP 2000F/300E) programmed with the following schedule: 10% neutral buffered formalin, 2 hours at 40°C; 70% industrial methylated spirit (IMS), 1 hour at 40°C; 90% IMS, 1 hour, 40°C; absolute IMS, 3 hours, 40°C; xylene, 4 hours, 40°C; paraffin wax, 3 hours 60°C. Tissues were embedded in paraffin wax utilising the Tissue-Tek III. The cast blocks were then left at room temperature to allow the wax to become hard. After the wax hardened, the cast blocks were

removed from embedding mould and stored in a dry place at room temperature until required.

The blocks were sectioned at 5μm using a microtome at room temperature. The sections were floated on warmed distilled water (45°C) to prevent creasing, mounted onto 2% 3-aminopropyltriethoxysilane (APES) coated slides (see Appendix I), and dried at 42°C for 30 minutes. The slides were placed in the incubator at 37°C for 2 days to firmly attach the sections to the slides and were stored in a slide box and placed at 4°C until required. Each block also had a corresponding Haematoxylin and Eosin (H&E) stained slide taken for comparison.

2.1.2.3 SERUM AND TISSUE FLUIDS

Blood samples were taken 12-24 hours prior to surgery by peripheral venous puncture and were immediately centrifuged at 2500x g for 15 min to remove the cells. The serum obtained was divided into aliquots and stored at -80°C. In parallel, a serum study was performed on a group of healthy volunteers (menstruating women with no history or evidence of ovarian disease or family history of cancer). Peripheral blood (approximately 7 ml) was drawn on a weekly schedule, over the course of a single menstrual cycle. An average of 4 samples was taken per patient. The serum samples were prepared and stored as described for patients undergoing surgery.

For fluid collection, after the surgical removal of any suspected benign or malignant cysts and ascites, the surgeon punctured the cyst capsule with an 18-gauge needle mounted on a 10 ml syringe. If a tumour contained more than one cyst, fluid was drawn from the dominant cyst. All fluids were then centrifuged at

2500x g for 15 min and the supernatants immediately aliquoted and stored at -80°C.

### 2.1.3 CLINICOPATHOLOGICAL PARAMETERS

The clinico-pathological data such as age at diagnosis, tumour type, grade and stage were obtained during the recruitment of the patients.

## 2.2 METHODS

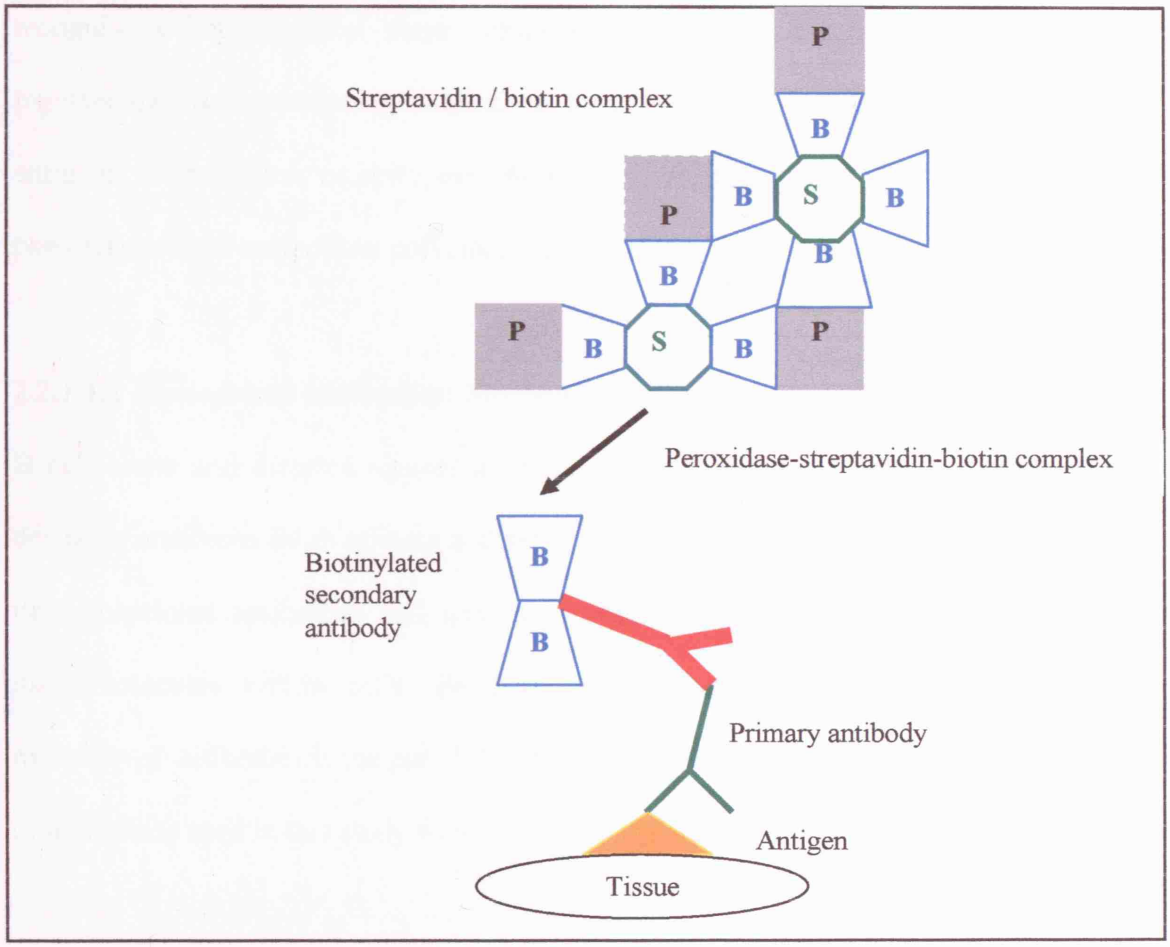
All procedures were performed using strict laboratory protocols that adhered to Control of Substances Hazardous to Health (COSHH) guidelines (October 1984).

### 2.2.1 IMMUNOHISTOCHEMISTRY (IHC)

H&E sections were examined by a consultant gynaecological pathologist (JCC) and myself to review the histopathological features.

IHC is the demonstration of antigens in tissue sections by the use of specific antigen-antibody interactions, which culminate in the attachment of a marker to the antigen. Through a series of steps, an enzyme that forms a coloured or fluorescent reaction product (visible with a microscope) is attached to the antibody 'probe,' allowing the presence or absence of the antigen in the cells to be determined as well as enabling an assessment of its distribution and variability within a given specimen to be made (Figure 2.1). This can be accomplished by viewing the stained slide with a standard or specialised microscope. IHC provides a permanent record with excellent preservation of cell morphology and can be performed on formalin-fixed paraffin wax-embedded tissue and on frozen tissue. IHC allows direct visualisation of the cells bearing or lacking the marker of

interest, so allowing direct correlation with histopathological features of the diagnostic material.




Abbreviations: B-Biotin; S-Streptavidin; P-horseradish peroxidase

**Figure 2.1 The Streptavidin-Biotin method for IHC.**

2.2.1.1 ANTIBODIES

Antibodies are typically produced in vertebrates as a defence against infection. They are produced by a class of white blood cells, called plasma cells which develop from B-lymphocytes or B cells. Briefly, antibodies can be made in the laboratory by injecting an animal (usually a mouse, rabbit, sheep or goat) with an antigen; for example, antigen A. Repeated injections of the same antigen at intervals of weeks stimulates specific B cells to develop into plasma cells and to secrete large amounts of anti-A antibodies into the bloodstream. As many

different B cells are stimulated by antigen A, the blood will contain a variety of anti-A antibodies, each of which binds A in a slightly different way. Antibodies recognise a 3-dimensional shape composed of discontinuous residues brought together into juxtaposition by folding of the molecule (Nelson et al, 1997). The antigenic determinants or epitopes usually comprise 6-8 amino acids. There are two categories of antibodies; polyclonal and monoclonal antibodies.

**2.2.1.1A Monoclonal antibodies:** Monoclonal antibodies are derived from single B cell clone and directed against a single epitope and hence display the most desirable attributes (high affinity and selectivity). They are usually more specific than polyclonal antibodies and serve as powerful tools in the investigation of macromolecules within cells (Blottiere et al, 1995). Another advantage of monoclonal antibodies is the possibility of background-free staining. The majority of antibodies used in this study were of monoclonal origin (Appendix I).

**2.2.1.1B Polyclonal antibodies:** Polyclonal antibodies are produced by different clones of cells and represent a family of antibodies that can react with various epitopes on the antigen against which they are raised.

2.2.1.2 IMMUNOLABELLING WITH STREPTAVIDIN-BIOTIN COMPLEX

Originally, the glycoprotein avidin was used to conjugate with biotin, however, avidin may bind non-specifically to negatively charged structures such as the nucleus, and as it is a glycoprotein, it can react with molecules such as lectins. In this study, the formation of streptavidin-biotin complex was chosen as the immunolabelling method of highest sensitivity because it uses its high affinity of

streptavidin for biotin. Streptavidin is a protein (molecular weight 60 kDa) isolated from the bacterium *Streptomyces avidinii,* and has 4 high affinity binding sites for biotin although not all are used due to molecular orientation. Streptavidin has an isoelectric point close to neutral pH and therefore possesses few strongly charged groups at the near neutral pH used in IHC detection systems. The physical properties of streptavidin make this protein much more desirable for use in IHC detection, compared to avidin. Streptavidin and biotinylated enzyme are simply mixed at appropriate concentrations and allowed to stand for at least 30 minutes at room temperature for the complex to form. This pre-formed complex is attached to the biotinylated secondary antibody. Careful stoichiometric control ensures that some binding sites remain free to bind with the biotinylated secondary antibody. This allows the pre-formed complex to bind and provides a very high signal at the antigen-binding site (Figure 2.1).

### 2.2.1.3 STANDARD IHC PROTOCOL

Immunohistochemical analysis for the expression of all the proteins in this work was performed using an indirect peroxidase-based labeling procedure with a streptavidin-biotin-horseradish peroxidase detection system (Dako, U.K, Appendix I). IHC was optimised to ensure reduction of non-specific background staining using a suitable positive control for each respective antibody. For IHC, sections were from formalin-fixed paraffin wax-embedded tissue.

The sections were deparaffinised in xylene and rehydrated in different grades of ethanol up to distilled water. Endogeneous peroxidase activity was quenched with a 3% v/v fresh solution of hydrogen peroxide followed by a wash in phosphate

buffered saline (PBS; see Appendix I). To optimise immunoreactivity, antigen retrieval was performed using different methods for each protein:

- **Heat-induced epitope retrieval (HIER)** – this method is used to increase the sensitivity of reactions directed to paraffin resistant antigens, especially those found in the nucleus. The most common HIER methods use pressure cookers, microwave ovens or autoclaves as the heat source and low molarity buffers with acid or alkaline pH (Cattoretti et al, 1993; Bankfalvi et al, 1994, Beckstead, 1994; Norton et al, 1994). In this project, several attempts using different conditions such as low to full power of the microwave or pressure cooker, heating incubation time of 2, 5, 10 and 15 minutes and different buffers (sodium citrate or EDTA buffer) were used to enhance the retrieval of the antigens. As a result, microwaving in sodium citrate buffer (pH 6.0, Appendix I) for 10 minutes at full power or pressure cooking in sodium citrate buffer (pH 6.0, Appendix I) for 2 minutes were the established optimum conditions.
- **Protease digestion or protease induced epitope retrieval (PIER)** – Initially, this method is commonly used to counteract the antigen masking effects of formalin fixation. It involves the use of numerous enzymes such as proteinase K, trypsin, DNAse and pepsin which is required for the cleavage of the molecular cross-linking, allowing the epitope to return to its normal configuration, hence enabling more binding of the antibody; however, the cleavage is not specific and some antigens might be negatively affected by this treatment (Batttifora et al, 1986). By trial and error, using different times incubation of 5, 10 and 15 minutes, as well as various concentrations of protease solution of 6, 10, 12.5 and 15 mg, the

optimised conditions were found to be the use of protease digestion (Bacterial protease Type 24, Sigma) 12.5 mg in 100 ml of PBS at 37°C for 10 minutes (Appendix I).

- **No antigen retrieval step** – this method is performed for antibodies which are easily detected, without causing any background that may result in non-specific staining.

The sections were then placed in a humidity chamber and a solution of serum (normal rabbit or goat) was added to prevent non-specific staining (10% v/v in PBS, left for 15-30 minutes at room temperature). This serum was then removed by tapping off the excess and the primary antibody was placed on top of the section using the optimum dilution (Table 2.1). All dilutions were performed in bovine serum albumin (BSA) diluted in PBS (see Appendix I) unless specified. Optimisation of antibody dilutions was performed for all primary antibodies on control tissue sections, initially using the dilution range recommended by the manufacturer and then performing other dilutions to optimise conditions. The sections from the control tissue were then assessed for intensity of staining and absence of non-specific staining. The optimal dilution chosen for each antibody was the one in which there was good quality brown staining which disappeared if the dilution was increased. Incubation times were also optimised for all antibodies, along with the incubation temperature, allowing specific staining with the lowest concentration of antibody but still providing intense specific staining. Table 2.1 shows the optimum antigen retrieval step used for each protein.

| **Antigen** | **Antigen retrieval** | **Primary antibody dilution** | **Incubation time for primary antibody** | **Incubation temperature for primary** |
|---|---|---|---|---|

| | | | | antibody |
|---|---|---|---|---|
| **IGF-1Ea** | None | 1:70 | Overnight | 4°C |
| **IGF-1Ec** | None | 1:400 | Overnight | 4°C |
| **TP** | Microwaving | 1:10 | 1 h | RT |
| **VEGF** | Protease digestion | 1:40 | Overnight | 4°C |
| **VEGF-C** | Pressure cooking | 1:40 | Overnight | 4°C |
| **VEGFR-1** | Microwaving | 1:10 | Overnight | 4°C |
| **vWF** | Protease digestion | 1:40 | 1 h | RT |

**Table 2.1 Optimum conditions used for each protein.**

Following incubation, the primary antibody was washed off in two 3-minute washes with PBS. The secondary antibody (Appendix I) was then applied in PBS-BSA containing a 10% dilution of normal human serum (see Appendix I). The secondary antibody uses the primary antibody as its antigen; hence a mouse monoclonal primary would have a secondary antibody raised against the mouse. This secondary antibody had been biotinylated; i.e. biotin had been added chemically. It was added at a dilution of 1:200 and was left at room temperature for 30 minutes. The secondary antibody was then removed by two 3-minute washes with PBS and then reacted with peroxidase-conjugated streptavidin-biotin (Appendix I) at a dilution of 1:200 for 30 min. Excess peroxidase was washed off in 2-3 washes of PBS and peroxidase activity was detected by incubating the sections in a solution of 3, 3'-diaminobenzidine tetrahydrochloride (Appendix I) for 5-8 min until a brown colour had developed. Placing the slides in tap water then terminated the reaction. The slides were counterstained in Mayer's haematoxylin, washed and finally rehydrated with grades of ethanol. They were then cleared in xylene to allow the refractive index to come to 1, mounted in DPX and a cover slip was then added.

2.2.1.4 CONTROLS

A known positive control was added to each staining batch to ensure that the staining was successful. The same control specimen was used for each run for a given antigen to evaluate the intensity of the stain. The control used was placental tissue in all cases. Negative controls were also included in each staining run. They were the tissue under investigation with omission of the primary antibody (replaced by PBS) in order to ensure that other reagents do not stain non-specifically. In the negative control for VEGF staining, the primary antibody was replaced by $IgG_2B$.

To ensure staining consistency and reproducibility, a number of precautions were taken:

i. Sections from different blocks were stained as a batch on the same day and the process was repeated 3-4 times for each protein to assess the uniformity of the intensity of the staining.

ii. If available, different blocks (approximately 2-3) from the same specimen were analysed separately and the most representative block was used.

iii. Results were analysed independently by three observers (Dr Chris Perrett, Dr Julie Crow and myself).

In all cases, there was <5% variation in results between sections, blocks and observers. A consensus score was then achieved.

2.2.1.5 STAINING ASSESSMENT

**2.2.1.5A: IGF-1Ea, IGF-1Ec, TP, VEGF-C and VEGFR-1 assessment**

Immunostaining for IGF-1Ea, IGF-1Ec, VEGF-C and VEGFR-1 was mainly cytoplasmic. TP immunostaining was both nuclear and cytoplasmic. We selected

three different fields in the middle of the tumour or in the middle of the stroma (for cases of normal ovarian samples) and the number of immunostained brown cells (positive) per hundred tumour cells (or stromal cells for normal cases) was estimated at X100 magnification. The results were classified as positive if 10% or more of the tumour (or stromal cells in cases of normal samples) were stained brown at x100 magnification (Table 2.2). To ensure consistency and reproducibility with regard to protein assessment, this threshold value was set at 10% for all five proteins.

| Antigens | % of positive cells | Score |
|---|---|---|
| **IGF-1Ea** | < 10% | Negative |
| | ≥10% | Positive |
| **IGF-1Ec** | < 10% | Negative |
| | ≥10% | Positive |
| **TP** | < 10% | Negative |
| | ≥10% | Positive |
| **VEGF-C** | < 10% | Negative |
| | ≥10% | Positive |
| **VEGFR-1** | < 10% | Negative |
| | ≥10% | Positive |

**Table 2.2 Assessment of IGF-1Ea, IGF-1Ec, TP, VEGF-C and VEGFR-1.**

### 2.2.1.5B: VEGF assessment

The whole section was visualised under a light microscope at low power initially as VEGF expression was highly focal in ovarian biopsies. The presence of VEGF was scored by counting the number of 'hot spots' or 'focal areas' i.e. clusters of brown cells representing VEGF per $cm^2$ of tissue section and scoring them accordingly. A section was scored positive if 10 or more focal areas were present

in the tumour areas (Gentry et al, 2001; Mullerat et al, 2005). A focal area consisted of 10 or more stained cells.

| No of focal areas representing VEGF per $cm^2$ of tissue section. | Score |
|---|---|
| < 10 focal areas | Negative |
| ≥ 10 focal areas | Positive |

**Table 2.3 Scoring system for VEGF.**

### 2.2.1.5C: Microvessel density

The method described by Weidner et al (1995) to score vWF staining in microvessels, was used by our group in ovarian tissue (Amis et al, 2005). The slides were first scanned at low magnification (X40 and X100) using a light microscope to identify areas containing the greatest number of stained vessels (hotspots) in both the stroma and tumour areas, excluding any area of necrosis. Any stained endothelial cell or endothelial cell cluster distinguishable from adjacent microvessels with or without a lumen was considered as a single countable microvessel. Distinct stained clusters that appeared to be from the same vessel snaking its way in and out of the section were considered distinct and countable as separate microvessels. Macrovessels with lumen larger than 50 μm in diameter (8 red blood cells) or with thick smooth muscular walls and widely distended venous sinuses, were excluded from the count. Taking these criteria into account, manual counting of vessels per optical field were assessed by 3 independent observers (JCC, CWP and myself). Three areas containing the highest number of hotspots at X100 magnification were firstly identified. These areas were then examined at X200 field (0.74 $mm^2$ under the light microscope) and at X200 field (0.17 $mm^2$ under the light microscope), and the highest vessel density (HVD) per $mm^2$ and average vessel density (AVD) per $mm^2$ of these three

fields were recorded. Previous experience by our group (Amis et al, 2005 and unpublished data) in similar histological areas of the female genital tract showed that there was no significant difference between using 3 or up to 10 hotspots per specimen to accurately assess angiogenesis. There was less than 5% variation in HVD/AVD assessment of each specimen between the 3 observers.

### 2.2.2 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA employs the quantitative enzyme immunoassay technique. Briefly, a monoclonal antibody specific, for example, to 'antigen A' has been pre-coated onto the bottom of the wells of a microplate. Standards and samples are then pipetted into the wells and any 'antigen A' present is bound to the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for 'antigen A' is added to the wells. Following a further wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and a colour develops in proportion to the amount of 'A' bound in the initial step. The colour development is stopped after a specified time period and the intensity of the colour is measured. A standard curve is constructed using supplied standards. All samples were assayed in duplicate and readings were made on a microplate reader (Anthos Lab Instruments, Austria). The microplate reader was set with the test filter set at 450 nm and correction wavelength set at 570 nm, to account for optical imperfections in the plate. Figure 2.2 shows a simplified version of the ELISA steps.

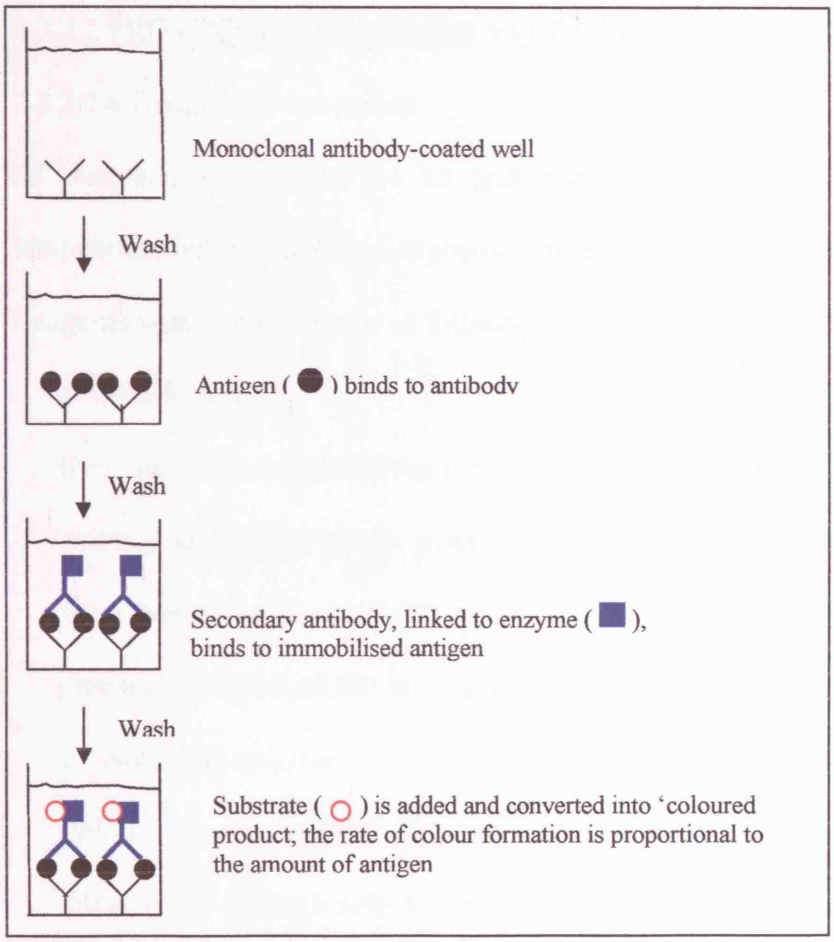



**Figure 2.2 Schematic representation of the ELISA method, in which the production of the colour indicates the quantity of antigen.**

2.2.2 1 COMMERCIALLY AVAILABLE KITS

In this study, two commercially available ELISA kits were used to measure the concentrations of VEGF and one of its receptors, VEGF-R2. Both kits were obtained from R&D Systems (Appendix I). The Quantikine Human VEGF kit has been used in many previous studies and has a low inter-and intra-assay error range (intra-assay error: 6.7-4.5, coefficient variation (CV) % in serum/plasma, inter-assay error: 8.8-6.2 CV% in serum/plasma). This assay is specific for VEGF and does not detect related molecules such as platelet-derived growth factor or PlGF. In contrast, the Quantikine Human VEGF-R2 kit has a low inter-and intra-assay

2.2.2.2 PROTOCOL FOR HUMAN VEGF ELISA

**2.2.2.2A Reagent preparation**

All reagents supplied in the kit and studied samples were brought to room temperature before use (the composition of each reagent is given in Appendix I). Reagents were then prepared as follows:

1. *Wash buffer*

If crystals had formed in the concentrate, the 'wash' buffer (Appendix I) was warmed and mixed gently until the crystals were completely dissolved. The wash buffer concentrate (20 ml) was then diluted into deionised water to prepare a solution of 500 ml of wash buffer (4% v/v).

2. *Substrate solution*

Colour reagents A and B (Appendix I) were mixed together in equal volumes (50% v/v) within 15 minutes of use and were protected from light. 200µl of the resultant mixture was required per well.

3. *VEGF standard*

The VEGF standard (Appendix I) was reconstituted with 1 ml of 'calibrator diluent' RD6U (Appendix I) to produce a stock solution of 2000pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making up dilutions. In a series of 6 polypropylene tubes, 500 µl of calibrator diluent RD6U was pipetted into each tube. The stock solution, used was the highest standard and was also used to produce the following dilutions (1000, 500, 250, 125, 62.5, and finally 31.2 pg/ml), all diluted in calibrator diluent RD6U. The latter was used as the zero standard or negative control (0 pg/ml).

**2.2.2.2B Assay procedure**

According to the manufacturer, the minimum detectable dose of VEGF is 9.0 pg/ml.

All samples, standards, and controls were assayed in duplicate.

1. All reagents and working standards were prepared as described in the previous section.
2. 100 µl of 'assay diluent' (Appendix I) was added to each well.
3. 100 µl of each standard, negative control or sample was then added to each well. In case the sample (particularly ascitic fluids) was too thick to pipette, a 1:2-1:5 dilution was carried out using calibrator diluent RD6U.
4. The wells, coated with a monoclonal antibody against VEGF and submerged with the standards or samples, were covered with the adhesive strip provided and left to incubate for 2 hours at room temperature. A plate layout was provided to record the standards and samples assayed.
5. Each well was aspirated and washed twice - each wash comprises 3 aspirations, with the wash buffer solution using an autowasher (Anthos Lab Instruments, Austria). The complete removal of liquid at each step was essential for good analysis and reproducibility. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.
6. 200 µl of VEGF conjugate (Appendix I) was added to each well and a new adhesive strip was placed on the microplate and left to incubate for another 2 hours at room temperature.
7. The aspiration and wash steps as in step 5 were then repeated.

8. 200 µl of substrate solution (Appendix I) was added to each well and again a new adhesive strip was used. The microplate was protected from light and incubated for 25 minutes at room temperature.
9. 50 µl of 'stop solution' (Appendix I) was added to each well. If the colour change did not appear uniform, the plate was gently tapped to ensure thorough mixing.
10. The optical density of each well was determined within 30 minutes, using a microplate reader set at 450 nm with a correction wavelength set at 570 nm, to account for optical imperfections in the plate and also to ensure well loading.

2.2.2.3 PROTOCOL FOR HUMAN VEGFR-2 ELISA

As with the previous kit, all reagents supplied and studied samples needed to be brought to room temperature before use.

**2.2.2.3A Reagent preparation**

As with the previous kit, the preparation of the 'wash buffer 'and 'substrate solution' (Appendix I) were common to both kits. The other reagents were as follows:

*1. VEGFR-2 standard*

The VEGFR-2 standard was reconstituted with 1.0 ml of deionised water to produce a stock solution of 50, 000 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making up dilutions. In a series of 7 polypropylene tubes, 900 µl of 'calibrator diluent' RD6-31 (Appendix I) was pipetted into one tube and 500 µl to the remaining tubes.

The stock solution (50,000 pg/ml) was used as the highest standard and was also used to produce a dilution series of 5000, 2500, 1250, 625, 312, 156 pg/ml, and finally 78 pg/ml, all carried out with calibrator diluent RD6-31. The latter was used as the zero standard (0 pg/ml).

*2. Samples*

Each studied sample was diluted 5-fold in calibrator diluent RD6-31 as recommended by the manufacturer. It is to be noted that some cystic and ascitic fluid samples studied in this project (see Chapter 5, section 5.4.3) were diluted to 1:2-1:5 in the dilution buffer (calibrator diluent RD6-31) because of their viscosity, making it difficult to be measured on the microplate reader. They were then re-multiplied by the dilution factor to restore the exact concentration.

**2.2.2.3B Assay procedure**

The assay procedure for VEGFR-2 was similar to VEGF, except that the substrate solution was incubated with the samples for 30 minutes. All reagents and their respective composition used in this assay are given in Appendix I. The minimum detectable dose of VEGFR-2 ranged from 1.0-11.4 pg/ml.

**2.2.3 REAL-TIME PCR (RT-PCR)**

The starting template for a PCR reaction can be DNA or RNA. DNA is usually the appropriate template for studying the genome of the cell or tissue (as in inherited genetic diseases, somatic mutation in a tumour, or somatic rearrangement in lymphocytes) and for the detection of DNA viruses (Wright and Wynford-Thomas, 1990). For information on gene expression in a cell (Telles et al, 2003)

or tissue (Santos et al, 2002; Garlet et al, 2003), or the presence of genomic RNA in a retrovirus such as HIV, RNA is the appropriate template. RNA can be a better template than genomic DNA for the detection of structural changes in long genes. The amplification of the spliced RNA transcript instead of the genomic DNA greatly reduces the length of DNA to be handled without losing any of the encoding regions where clinically significant deletions may be expected (Wright and Wynford-Thomas, 1990). RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method or the analysis of gene expression (see Figure 2.3).

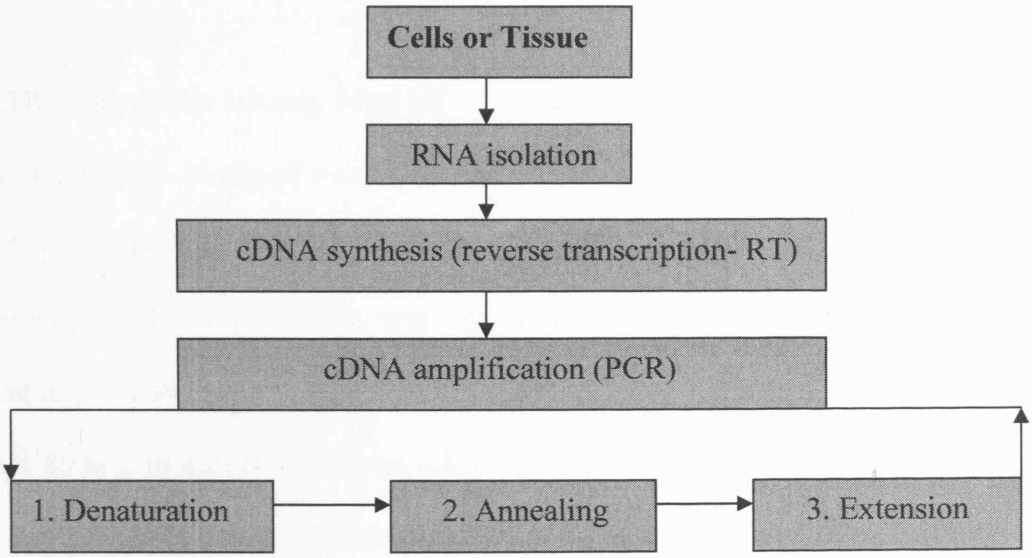



**Figure 2.3 Schematic diagram of RT-PCR.**

2.2.3.1 RNA ANALYSIS

Compared to DNA, RNA is relatively unstable. This is due to the presence of ribonucleases (RNAases), which break down RNA molecules. RNAase contamination can come from the human skin and dust particles, which can carry bacteria and moulds. Isolation and analysis of RNA therefore requires specialised techniques to be carried out using sterile equipment and sterile reagents.

Therefore, as a precaution, all RNA samples were treated with RNase-free DNase water during the RNA extraction step.

### 2.2.3.1A: Extraction of RNA from frozen tissues

Total cellular RNA was extracted from snap frozen ovarian samples using the RNeasy Mini kit (Qiagen), as described in the next paragraph, according to the manufacturer's instructions. The method is based upon the isolation method developed by Chomczynski and Sacchi (1987) using guanidinium isothiocyanate, β-mercaptoethanol, phenol and chloroform.

### 2.2.3.1B Protocol for RNeasy Mini kit

Approximately 25-30 mg of tissue was homogenised (Polytron) at high speed in Tri-Reagent containing β-mercaptoethanol (Appendix I) before extraction. It was important that the frozen tissue was not allowed to thaw during handling to prevent any destabilisation of the tissue. The tissue lysate was then centrifuged (3 min) at 8000 g in a microcentrifuge tube. Only the supernatant (lysate) obtained was used in subsequent steps. Ethanol (70% v/v) was then added to the cleared lysate to adjust binding conditions and it was mixed immediately by pipetting. The sample was transferred to an RNeasy mini column (Appendix I) placed in a 2 ml collection tube for adsorption of RNA to the membrane. Contaminants were removed with 3 simple wash spins of 5 seconds, with buffers RWI and RPE (Appendix I) in succession. Finally, the extracted RNA was dissolved in 1% diethylpyrocarbonate-treated (DEPC) water and quantified spectrophotometrically at 260 nm (Gene Spec I, Naka Instruments, Japan). The quality of the RNA was assessed on a denaturing formaldehyde agarose gel (2%) by visual inspection after

ethidium bromide staining of the 18S and 28S rRNA bands under ultra-violet light.

**2.2.3.1C: DNAse treatment of RNA**

Total RNA was treated with RQ1 RNase-free DNase (Promega) to remove any contaminating genomic DNA that may interfere with later PCR amplification steps. 1µg RNA was treated per reaction consisting of 1-8µl RNA in $H_2O$, 1µl 10x reaction buffer (400mM Tris-HCl (pH 8.0), 100mM $MgSO_4$ and 10mM $CaCl_2$), 1µl RQ1 DNase (1 unit/µl) and nuclease-free water to a final volume of 10µl. The reaction mixture incubated at 37°C for 30 minutes before adding a stop solution (20mM EGTA, pH 8.0) and heat deactivating the DNase at 65°C for 10 minutes.

2.2.3.2 cDNA SYNTHESIS

Total RNA was reverse transcribed into cDNA in two steps, using Omniscript reverse transcriptase (Qiagen). The Omniscript kit for reverse transcription used is specially designed for reverse transcription using 50 ng to 2 µg per reaction. First, a quantity (0.5 µg) of total RNA was mixed with DEPC treated water (1%) in a total volume of 10 µl and heated to 65°C, to denature any secondary RNA structure, for 5 minutes before transferring to ice. Second, the samples were then mixed to the PCR mixture prepared on ice as follows:

- 10X First Strand buffer 2 µl
- 5 mM deoxynucleotide triphosphates (dNTPs) 2 µl
- 15 pmol/l Sequence specific primer for IGF-1Ea and IGF-Ec 1 µl
- 50 pmol/l Random hexamer primer for VEGF 1 µl
- 10 units/µl RNase inhibitor 1 µl

- 4 units/μl Omniscript reverse transcriptase 1 μl
- Double distilled water to 20 μl

The samples were then incubated at 37°C for 1 hour followed by 5 minutes at 93°C to inactivate the reverse transcriptase. The sequence for the specific RT primer used for IGF-1Ea and IGF-1Ec analysis was; 5'-GAAACGCCCATC-3'. For every reaction set, one RNA sample was performed without the Omniscript reverse transcriptase (RT-minus reaction) to provide a negative control in the subsequent PCR. To minimise variation in the reverse transcription reaction, all RNA samples from a single setup were reverse transcribed simultaneously. The use of larger amounts of cDNA in the PCR was avoided, since this may inhibit efficient PCR amplification.

**2.2.3.2A Designing primers and probes**

The oligonucleotide primers used for real-time qRT-PCR were designed using the computer program Omiga version 2.0 software (Oxford Molecular, UK). The default parameters of this program are set very narrow; most important are the melting temperature (Tm) of the primers and probe, and the amplicon length. The Tm of the primers is 58-60°C (see Table 2.4) while the Tm of the probe should be at least 10°C higher, approximately 68-70°C, to anneal to the target sequence during the extension step of the PCR (PCR extension is performed at 60°C). An advantage of these narrow temperatures is that the temperature cycle conditions for PCR amplification are identical to all targets.

| Target | Primer sequences | Product size (bp) | Tm (°C) | Cycles |
|---|---|---|---|---|
| **VEGF** | U: GCAGAATCATCACGAAGTGG<br>D: GCATGGTGATGTTGGACTCC | 212 | 58 | 45 |
| **IGF-1Ea** | U: GCCTGCTCACCTTCACCAGC<br>D: TCAAATGTACTTCCTTCTGGGTC | 303 | 60 | 40 |
| **IGF-1Ec** | U: CGAAGTCTCAGAGAAGGAAAG<br>D: TCAAATGTACTTCCTTCTGGGTC | 150 | 60 | 40 |

**Table 2.4 Primers, product sizes and reaction conditions used for qRT-PCR.**

For each target gene, different concentrations of $MgCl_2$ (1-5 mM) and primers (100-900 nM) were tested to optimise the PCR. In this study, 1.5 mM of Mg+ and 10 pmol/l of primer concentration were optimal for PCR amplification.

At present, the use of a 'housekeeping gene' is currently the most acceptable method to correct for minor variations due to differences in input RNA amount or in efficiencies of reverse transcription. An ideal housekeeping gene should be expressed at a constant level among different tissues of an organism, at all stages of development, and should not be affected by the experiment itself. Housekeeping genes like α actin and GAPDH are widely used to normalise results in RT-PCR. In this study, GAPDH - an abundant glycolytic enzyme in most cell types - was used as the endogenous control, although several recent studies have severely criticised GAPDH expression to be influenced by some experimental conditions (Barroso et al, 1999; Freeman et al, 1999; Bustin; 2000; Suzuki et al, 2000).

2.2.3.3 QUANTITATIVE REAL-TIME RT-PCR (qRT-PCR)

**2.2.3.3A Principle of qRT-PCR**

Quantitative RT-PCR is based on the detection and quantification of a fluorescent emitted during amplification of each PCR cycle (i.e., in real time). This fluorescent signal (SYBR in this case) increases in direct proportion to the amount of PCR product in a reaction. Owing to the real-time detection of fluorescent signals during and/or after each subsequent PCR cycle, quantitative PCR data can be obtained in a short period of time. At present three main types of qRT-PCR techniques are available: RT-PCR analysis using SYBR Green I dye, RT-PCR analysis using hydrolysis probes such as TaqMan probe or double oligonucleotide probe and RT-PCR analysis using hybridisation probes such as fluorochromes.

**2.2.3.3B SYBR Green I dye**

The simplest and commonest RT-PCR technique is based on the detection of PCR products by DNA-intercalating dye SYBR Green I (Figure 2.4). The SYBR Green I is a dye-binding dye that incorporates into double stranded DNA. It has an undetectable fluorescence when it is in its free form, but once bound to the double stranded DNA, it starts to emit fluorescence. During consecutive PCR cycles, the amount of double stranded PCR product will potentially increase, and therefore more SYBR Green I dye can bind and emit its fluorescence (at 520 nm). The fluorescence signal will gradually increase during the extension phase, will be maximal at the end of each extension phase, and will be low or absent during the denaturation phase. Its greatest advantage is that it can be used with any pair of primers for any target, making it less expensive than that of a probe.

denaturation phase. Its greatest advantage is that it can be used with any pair of primers for any target, making it less expensive than that of a probe.

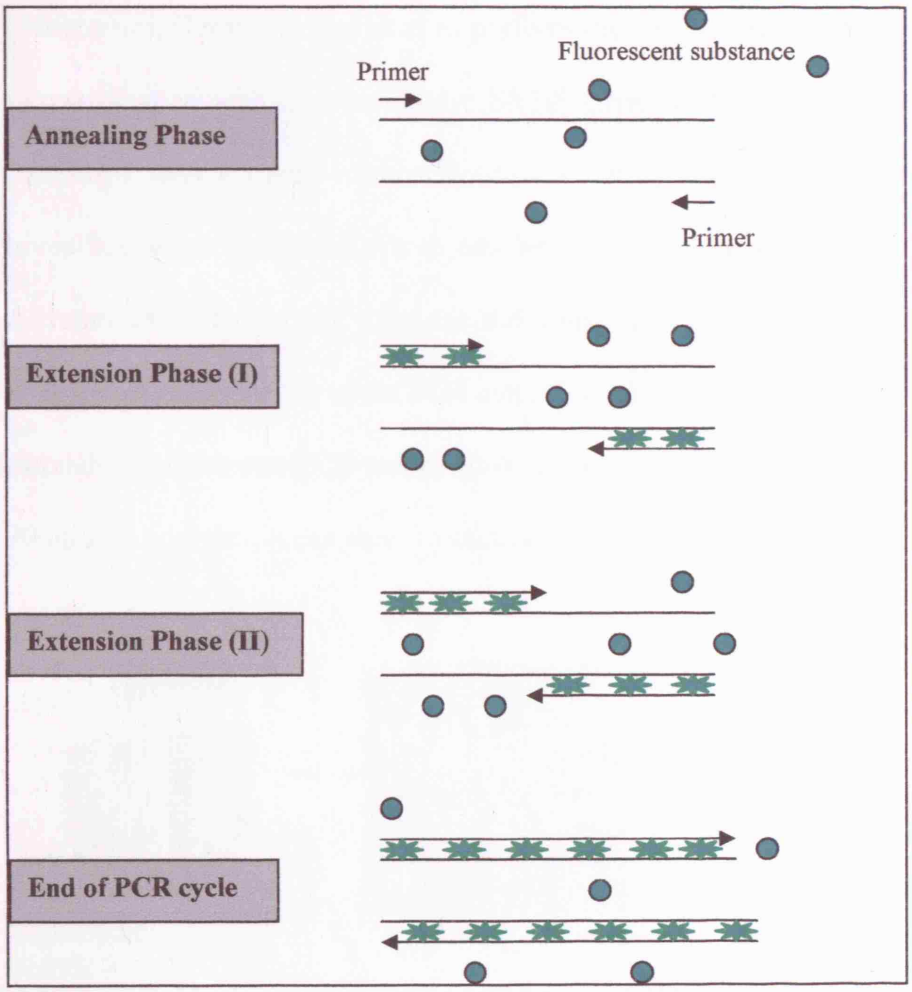
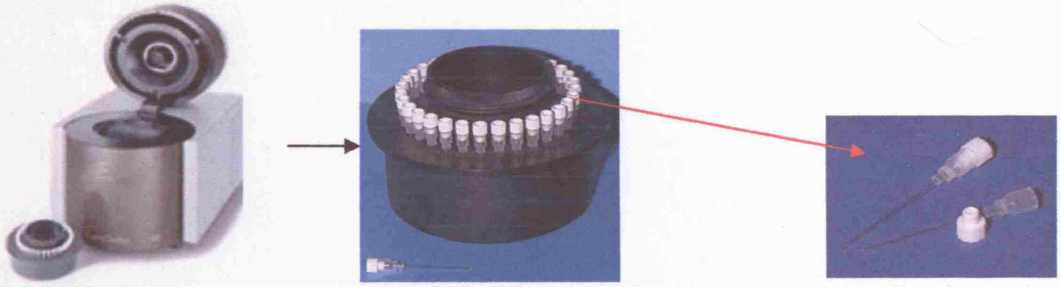



**Figure 2.4 Schematic diagram of SYBR Green I method.**

However, specificity is diminished due to the risk of amplifying non-specific PCR products (Simpson et al, 2000). Indeed, SYBR Green I binds to any double stranded DNA, detecting not only the specific target, but also non–specific PCR products and primer dimers. This is a common disadvantage. There are a few ways of handling this problem, the comparison of Tm being one of them and it was used in this study. The LightCycler is capable of analysing melting curves of the reaction and this will be discussed in the section that follows.

### 2.2.3.3C The LightCycler

To my knowledge, at least seven qRT-PCR instruments are commercially available. In this study, the LightCycler from Roche Molecular Biochemicals (Mannheim, Germany) was used to perform qRT-PCR, since it is most often used in combination with the binding dye SYBR Green I. It is a rapid thermal cycler, combined with a micro-volume fluorimeter using high quality optics and uses borosilate glass capillaries which can hold up to 20 µl of sample (Figure 2.5). Advantages of the LightCycler are the ability to view and analyse the data by monitoring fluorescence while PCR amplification is still in progress, and the high throughput, since one PCR run of 30-40 cycles can be completed in less than 20-30 min. In addition, it can detect mutations by Tm analysis.



LightCycler Carousel holding capillaries that are used as reaction vessels

**Figure 2.5 The LightCycler**

The LightCycler instrument is then linked to a computer in which the software displays the fluorescence signals in real-time immediately after each measurement. To evaluate whether specific PCR products have been formed, a melting curve analysis can be performed. In such analysis, the temperature is slowly increased from 40 to 95°C with continuous monitoring of the fluorescence. Fluorescence will be high at low temperatures when all DNA will be double stranded, but will drastically decrease around the melting temperature (Tm) of the

DNA products. PCR products of different length or sequence will melt at different temperatures and will be observed as distinct peaks. Once the melting curves are established, it is possible to set the software, to acquire fluorescence above the primer dimers' melting temperature but below that of the product. If only the specific PCR product has been formed, only a single peak should be visible in the melting peak profile.

**2.2.3.3D The protocol**

The oligonucleotide primers were synthesised by Sigma Genosys (Cambridge, UK). They were received in a dessicated form, reconstituted to 100 pmol/l with DNAse free water, aliquoted and stored at -20°C. Before use, one aliquot was diluted to a concentration of 10 pmol/l, stored at 4°C and used when required. In this study, two primers were used for each gene analysed - one upstream (U) and one downstream (D), and their sizes are listed in Table 2.4.

Briefly, quantitative PCR was performed in a total reaction volume of 20 µl per capillary for the LightCycler format. This reaction mix contained 10 µl of a SYBR green mix (QuantiTect, Qiagen, UK), 0.5-10 pmol/l of each forward and reverse primer, 2 µl cDNA (made from 0.5 µg RNA) and nuclease-free water to make up the reaction volume. The cDNAs were amplified initially by denaturation at 96°C for 5 min, followed by various cycles of denaturation at 94°C for 1 min, annealing (60°C for 1min), and extension (72°C for 2 min). Serial dilutions of standard DNA (usually any cDNA sample expressing the target sequence of interest) of 'known' concentration were included in each run from which a standard curve was created (Appendix I). It was relative to this standard curve that samples of unknown concentration were quantified. On completion of the PCR, all PCR products

formed were melted to attain a melting curve profile which enabled the specificity of the reaction to be determined (Appendix II). Runs were performed in duplicate and mean values were subsequently used for analysis. To ensure unbiased analysis, qRT-PCR was carried out blind and the identity of the samples was only revealed after the mRNA measurements had been made.

## 2.3 STATISTICS

For the statistical analysis the data were numerically coded and transferred to a Microsoft Excel (2003) spread sheet and analysed using the statistical package SPSS for Windows, version 10, Chicago, IL. All clinical data (age, tissue and histological subtype) were analysed along with data for VEGF, VEGF-C, VEGFR-1, VEGFR-2 and TP as well as microvessel density. The data was either continuous (e.g. age, VEGF expression) or categorical (tissue and histological subtype). For age, MVD, fluid levels, means ± standard deviation (SD), and medians, were recorded as appropriate. A level of $P<0.05$ was considered as statistically significant.

### 2.3.1 CHI-SQUARED ($\chi^2$) TEST AND FISHER'S EXACT TEST

$\chi^2$ is used to evaluate the presence or absence of a nominal outcome. The aim is to analyse 2 or more groups to compare the success rate and the failure rate in each group. The Chi-squared test was applied to assess the relationship between different categorical clinicopathological parameters (e.g. comparison of VEGF positive cases between benign ovarian cystadenomas and controls). If there are non-random associations between two categorical variables, Fisher's exact test is performed (e.g. comparison of VEGF positive cases between EOC subtypes).

### 2.3.2 MANN-WHITNEY *U* AND KRUSKAL-WALLIS *H* TESTS

The Mann-Whitney *U* or Kruskal-Wallis *H* tests were used where a non–parametric distribution was assumed. The Mann-Whitney *U* test was used for pairwise comparisons between two groups and the Kruskal-Wallis *H* test for more than two groups (e.g. comparison of several protein markers).

### 2.3.3 INDEPENDENT SAMPLES STUDENT'S T TEST

The Independent samples T test was used to compare the mean scores of two groups on a given variable (e.g. comparison of mean MVD values between two groups of tissues).

# CHAPTER THREE

# ANGIOGENESIS IN BENIGN OVARIAN LESIONS

**Abstract:**
Quantification of vascular endothelial growth factor-A (VEGF-A) and platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) in normal, benign and malignant ovarian lesions.
**Wong Te Fong LF**, Gammell SJ, Bamberger ES, Reid WMN, Crow JC, MacLean AB, Perrett CW.
**Revista de Oncologia 2002; 4 Suppl 1:1-179**

**Poster Presentation:**
Quantification of vascular endothelial growth factor-A (VEGF-A) and platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) in normal, benign and malignant ovarian lesions.
**Wong Te Fong LF**, Gammell SJ, Bamberger ES, Reid WMN, Crow JC, MacLean AB, Perrett CW.
**17th Meeting of the European Association for Cancer Research, June 2002, Granada Spain.**

## 3.1 INTRODUCTION

The terms 'ovarian tumour' and 'ovarian neoplasm' are often used interchangeably. It is now generally accepted that the terms 'tumour' and 'neoplasm' are synonymous. Any current definition must now recognise that all neoplastic proliferations have some abnormality in their DNA, whether it is obviously aneuploid or only slightly abnormal by virtue of a minor DNA alteration influencing the expression of an oncogene or tumour suppressor gene (Seidman and Mehrotra, 2005).

Epithelial ovarian neoplasms can be categorised into three distinct groups, depending on their behaviour – cystadenomas, borderline tumours and cystadenocarcinomas / ovarian carcinomas.

### 3.1.1 BENIGN CYSTADENOMAS AND BORDERLINE TUMOURS

Cystadenomas are benign ovarian tumours with no potential to metastasise or recur. It is thought that metaplasia of the surface epithelium of the ovary may be the starting point of a transformation that leads to the serous, mucinous or endometrioid cystadenomas and even cystadenocarcinomas, in a similar way that squamous metaplasia of the cervix may precede cervical intraepithelial neoplasia and invasive cervical malignancy. Puls et al, found that out of 94 ovarian carcinomas, 56% of serous and 90% of mucinous cystadenocarcinomas contained areas of histologically benign epithelium. They deduced that although the presence of benign epithelium could indicate that benign epithelium is found within malignant ovarian tumours, it was equally plausible that the malignant tumour arose from a benign neoplasm. They therefore postulated that a certain percentage of benign cystic tumours could undergo neoplastic transformation

(Puls et al, 1992). Zheng et al found that ovarian carcinoma expressed p53, while benign and borderline tumours do not. However, they also showed that benign cystadenomas of the ovary adjacent to malignancy expressed p53, suggesting that these tumours were atypical and carried a genetic predisposition to carcinogenesis (Zheng et al, 1995). Others have found that dysplasia and hyperplasia in the ovarian surface epithelium or in the inclusion cysts are potential histological precursors to ovarian cancer (Salazar et al, 1996; Werness et al, 1999). However, Bell and Scully have suggested the origin of ovarian cancer being directly from the surface of the epithelium and not preceded by a benign or borderline tumour (Bell and Scully, 1994).

Borderline tumours of the ovary, also known as tumours of low malignant potential, generally behave like the benign cystadenomas. Borderline tumours cannot metastasise because they are not invasive. They are however sometimes associated with peritoneal 'implants' which can be non-invasive or invasive but these are more likely to be 'field change' lesions similar to those arising from the ovarian surface. Unlike serous borderline tumours, mucinous, endometrioid and clear cell borderline tumours are often associated with their corresponding carcinomas. These tumours except serous type represent an intermediate stage in the stepwise sequence progression to ovarian cancer. Serous borderline tumours are thought to give rise to low grade serous carcinomas whereas high grade serous carcinomas do not go through this progression (Gilks et al, 2004). However, in mucinous carcinoma, for example, an increasing frequency of *KRAS* oncogene mutation has been described in cystadenomas, borderline tumours and mucinous carcinomas respectively (Enomoto et al, 1991; Mok et al, 1993; Ichikawa et al, 1994; Caduff et al, 1999; Gemignani et al, 2003) and using micro-dissection, the

same *KRAS* oncogene mutation has been detected in mucinous carcinoma and in adjacent mucinous cystadenoma and borderline mucinous tumour (Mok et al, 1993). Similarly, an increasing frequency of β-catenin mutation (Moreno-Bueno et al, 2001; Wu et al, 2001) and PTEN mutation (Obata et al, 1998) has been demonstrated in endometriosis with endometrioid adenofibromas, endometrioid borderline tumours and endometrioid carcinomas. Moreover, similar molecular genetic changes including mutations in PTEN and loss of heterozygosity (LOH) at 10q23 have been reported in different stages of tumour progression in the same specimen (Obata et al, 1998; 2000; Bischoff et al, 2000; Thomas et al, 2000; Sato et al, 2000). Like endometrioid tumours, clear cell borderline tumours are also frequently associated with endometriosis, clear cell adenofibromas and clear cell carcinomas, but molecular evidence for the stepwise progression is lacking, because molecular markers specific to clear cell neoplasms have only recently been identified (Hough et al, 2000; Tsuchiya et al, 2003).

### 3.1.2 ENDOMETRIOSIS

Endometriosis, defined as the presence of endometrial glandular and stromal cells outside the uterine cavity, is a benign gynaecological disorder that is usually identified at laparoscopy and is localised to ovaries and the pouch of Douglas. It is associated with significant pain and morbidity occurring in approximately 10% of women of reproductive age and in up to 50% of women with infertility (Strathy et al, 1982), however, its pathogenesis is still poorly understood.

Malignant transformation of endometriosis was first described by Sampson in 1925. Recent evidence from other authors reported approximately 60-80% of cases of endometriosis-associated ovarian cancer occur in the presence of atypical

ovarian endometriosis (Fukunaga et al, 1997; Ogawa et al, 2000; Oral et al, 2003). Of these cases, 25% show direct continuity of the atypical ovarian endometriosis with ovarian cancer (Fukunaga et al, 1997), underlying a potential 'pre-malignant transition spectrum of non-atypical to atypical and malignant variants.

Clear-cell and endometrioid carcinomas are the commonest endometriosis-associated ovarian cancers with ovarian endometriosis, while clear-cell adenocarcinoma and adenosarcoma are the commonest endometriosis-associated ovarian cancers in extra-ovarian endometriosis (Erzen and Kovacic; 1998; Stern et al, 2001; Zaino et al, 2001). The risk of direct malignant transformation of ovarian endometriosis has been estimated as 0.7-1.6% over an average of 8 years (Seidman 1996; Nishida et al, 2000). Jiang et al have shown that a proportion of cases of ovarian endometriosis exhibit LOH at one or more loci on chromosome arms 5q, 6q, 9p, 11q and 22q (Jiang et al, 1996; 1998) and that in cases with ovarian endometriosis adjacent carcinoma, common LOH events are present in the endometriotic and carcinomatous areas, suggesting a possible link for both with direct progression of endometriosis to carcinoma (Jiang et al, 1998). However, the precise genetic events underlying neoplastic change in endometriosis remain unknown, although it is possible that mutations in PTEN tumour suppressor gene may play a role (Obata et al, 1998).

### 3.1.3 ANGIOGENESIS

Angiogenesis, defined as the outgrowth of new capillaries from pre-existing vessels, is a development process that occurs during embryogenesis and is down-regulated in the healthy adult (Risau, 1995). In adults, angiogenesis is linked to physiological conditions such as ovarian and endometrial alterations during the

menstrual cycle. On the other hand, angiogenesis is also associated with the progression of hyperplasia and neoplasia (Folkman, 1971).

A number of studies have investigated the possible link between angiogenesis and pre-malignant lesions of breast, colon, cervix and vulva before the onset of frank invasion (Guidi et al, 1994, 1995; Bossi et al, 1995, Dobbs et al, 1997; MacLean et al, 2000). For example, work by our group showed that VEGF - a key angiogenic factor and regulator of endothelial cell function - is not expressed in normal vulval lesions, but is expressed in 6% of vulval intraepithelial neoplasia (VIN) lesions and 92% of vulval cancers (MacLean et al, 2000). Similar results were confirmed by myself (Wong Te Fong et al, 2000). This suggests that VEGF may be involved in malignant progression in the vulva and may be a valuable marker of premalignant lesions which go on to become invasive.

Another well-recognised inducer of angiogenesis is TP. It has been shown that TP may contribute to both the acceleration of angiogenic activity in the early process of invasion of well differentiated tumours in the breast (Fox et al, 1996), endometrium (Fujimoto et al, 1998), lung (Giatromanolaki et al, 1997), gastric (Maeda et al, 1996), colon (Takebayashi et al, 1996), uterine cervix (Fujimoto et al, 1999), and bladder (O'Brien et al, 1996). In addition, for example in endometrial cancer, Seki et al reported that that MVD (a risk factor for vascular invasion) was significantly correlated with the level of VEGF and TP mRNA expression and that TP expression is associated with increased MVD, suggesting that these growth factors may promote tumour invasion and progression at the invading tumour front (Seki et al, 2000).

In the literature, data concerning VEGF, TP activity with MVD and its correlation with neoplastic angiogenesis in primary EOC are still unclear. Study of VEGF

expression combined with other angiogenic markers, in relation to proliferation in ovarian tumours, may therefore help to find out whether the process of angiogenesis may be a 'propelling' force of pre-malignancy to EOC. In this study, the angiogenic markers selected to be investigated are VEGF, VEGF-C (a lymphangiogenic factor), VEGFR-1 (involved in VEGF signalling) and TP. MVD is used as an indirect measure of angiogenesis.

## 3.2 AIMS

The aims of this chapter were as follows to:

- perform IHC to examine the expression of several angiogenic proteins (VEGF, VEGF-C, VEGFR-1 and TP) and MVD in a range of benign ovarian lesions compared to normal ovaries,
- investigate the relationship between benign lesions and controls with respect to histopathological features and diagnosis, and
- clarify the clinical relevance of these markers in relation to ovarian pre-malignancy.

## 3.3 MATERIALS AND METHODS

### 3.3.1 MATERIALS

The study concerned 55 patients diagnosed with benign ovarian lesions (see Table 3.1) who underwent either hysterectomy or unilateral / bilateral salpingo-oopherectomy, either at the Royal Free or North Middlesex Hospitals, London, during 1991-2001, and 24 normal ovarian specimens obtained from women who had undergone oophorectomy for non-ovarian conditions (e.g. leiomyoma, uterine bleeding, pelvic pain).

| Diagnosis | n | Source |
|---|---|---|
| Normal ovarian tissues (control group) | 24 | Royal Free Hampstead NHS Trust Hospital |
| Benign ovarian cystadenomas | 18 | Royal Free Hampstead NHS Trust Hospital |
| Endometriosis | 27 | North Middlesex Hospital |
| Borderline ovarian tumours | 10 | Royal Free Hampstead NHS Trust Hospital |

**Table 3.1 Benign ovarian neoplasms.**

Five μm serial sections from formalin-fixed paraffin wax-embedded tissue samples were prepared. A section from each tissue was stained with H&E and re-evaluated by a consultant gynaecological pathologist (JCC) to confirm the original diagnosis. Histological types and grades of tumours were determined, where appropriate, using the WHO criteria (Serov et al, 1973) and are given in Table 3.2.

| Number of tumours | n |
|---|---|
| **Cystadenomas** | |
| **Serous** | 7 |
| **Mucinous** | 11 |
| **Borderline tumours** | |
| **Serous** | 4 |
| **Mucinous** | 4 |
| **Endometrioid** | 2 |

**Table 3.2 Histological characteristics of benign ovarian specimens.**

### 3.3.2 IMMUNOHISTOCHEMISTRY (IHC)

IHC was performed to detect the protein expression of VEGF, VEGF-C, VEGFR-1 and TP, as described in Chapter 2, Section 2.2.1.3 using the antibodies as shown in Table 2.1. The blood vessels in the paraffin wax-embedded tissue sections were highlighted by immunohistochemical staining of endothelial cells with anti-vWF

antibody. IHC was repeated three times to ensure consistency and scoring of each marker was as described in Section 2.2.1.4 and 2.2.1.5 respectively.

### 3.3.3 STATISTICAL ANALYSIS

Expression of these angiogenic proteins and MVD in relation to various clinicopathological factors were assessed using the chi-square test and Fisher's exact test. Correlation between expression of the angiogenic proteins and MVD was examined by the Spearman rank correlation test. A p value of $<0.05$ was accepted as statistically significant.

## 3.4 RESULTS

The mean age for all the patients, in each category, is given in Table 3.3 where no statistical significant difference in the mean age of the 3 different groups versus controls was found.

| **Diagnosis** | **n** | **Age in years mean ± SD (range)** | **p value (vs controls)** |
|---|---|---|---|
| **Controls** | 24 | 49.8 ± 11.3 (25-81) | |
| **Benign cystadenomas** | 18 | 46.9 ± 16.5 (24-89) | 0.50 |
| **Endometriosis** | 27 | 44.7 ± 7.3 (36-73) | 0.55 |
| **Borderline tumours** | 10 | 54.5 ± 14.3 (29-75) | 0.32 |

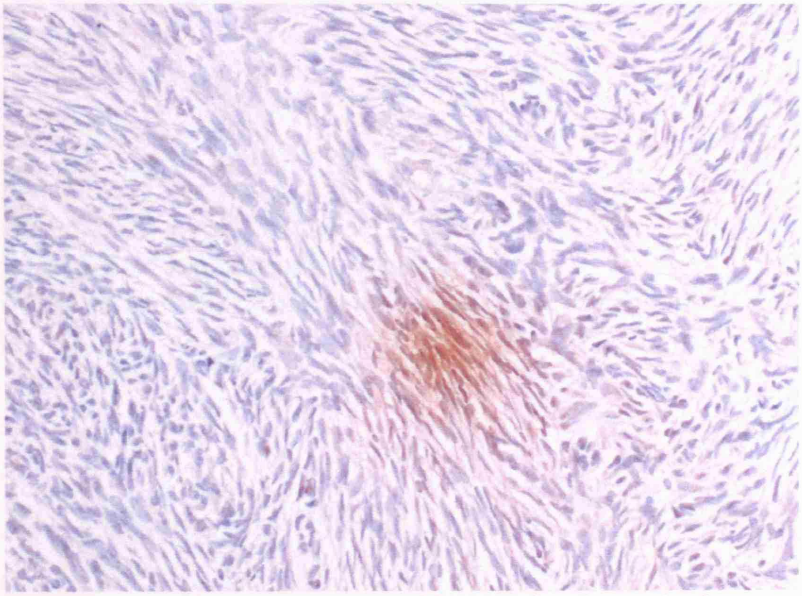
**Table 3.3 Mean age for each tissue type.**

Each protein marker was then assessed individually for all three groups of benign ovarian conditions.

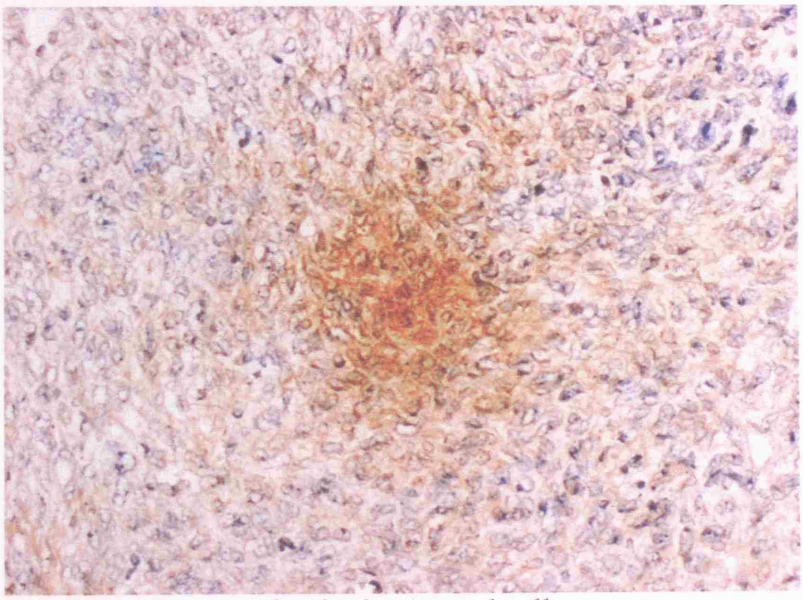
### 3.4.1 VEGF EXPRESSION

#### 3.4.1.1 VEGF IN OVARIAN TISSUES

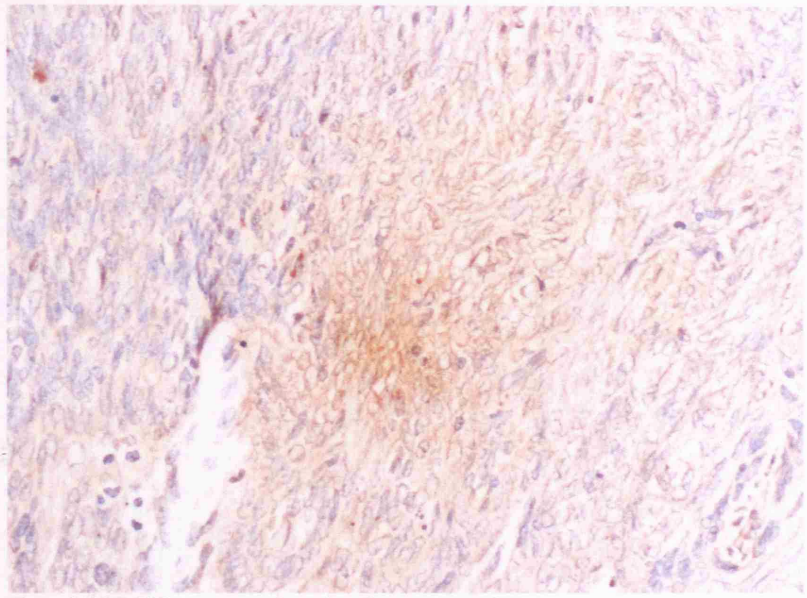
Positive VEGF staining was observed in both the cytoplasm and nuclei of stromal cells (Figure 3.1a-e). In borderline ovarian tumours, positive VEGF staining was found in both epithelial and stromal cells of the tumour (Figure 3.1e-f).



VEGF expression as represented by one focal area in the stroma.
**Figure 3.1a VEGF in normal ovary (X200).**

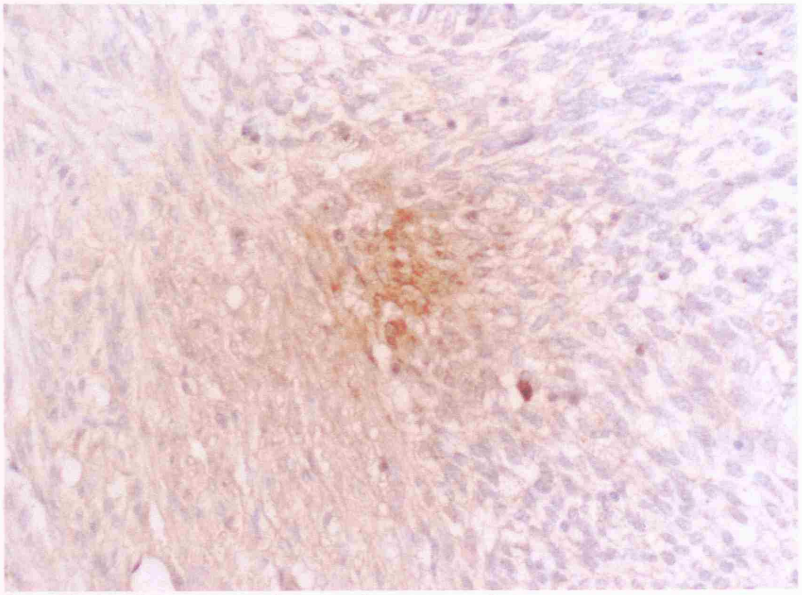


Focal VEGF expression in the stromal cells.
**Figure 3.1b VEGF in serous ovarian cytadenoma (X200).**



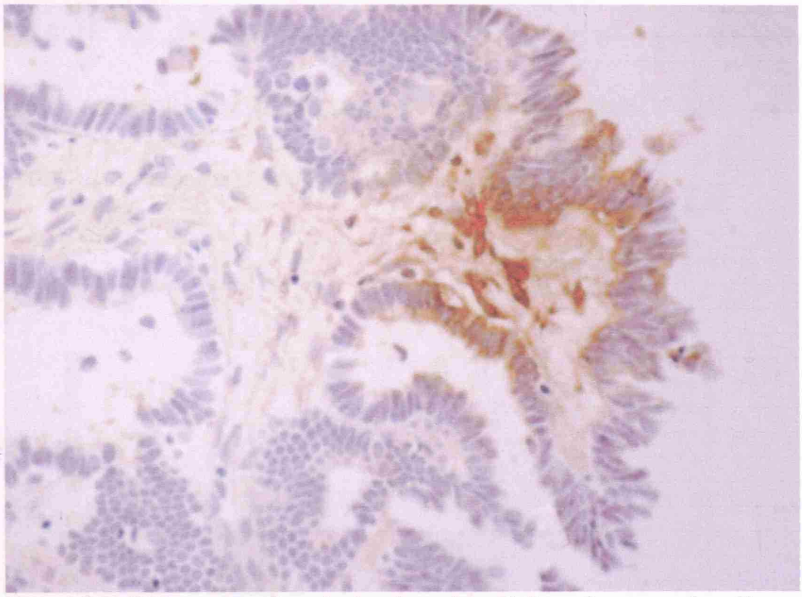
Focal VEGF expression in the stromal area.

**Figure 3.1c VEGF in mucinous ovarian cystadenoma (X200).**

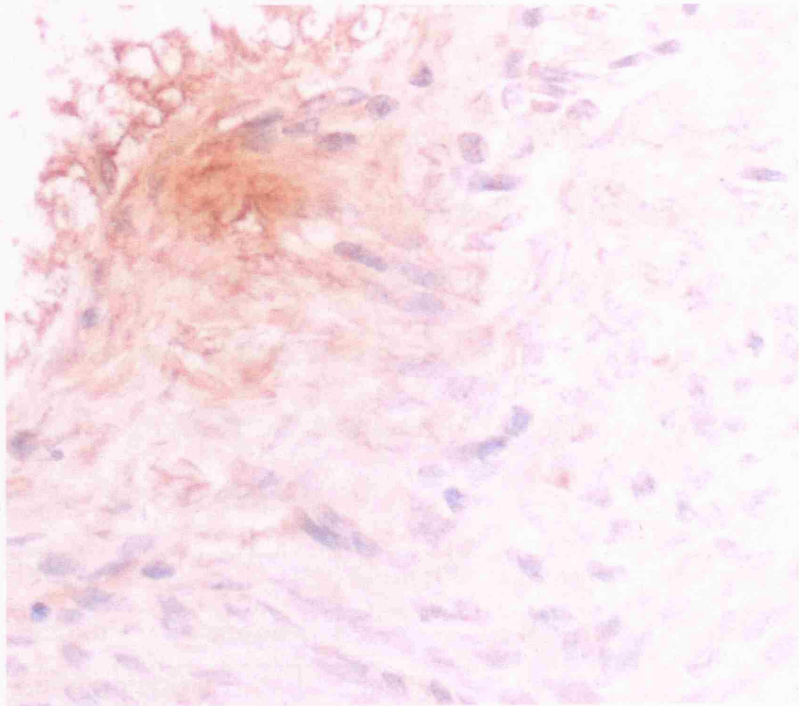


Focal VEGF expression in the stromal area.

**Figure 3.1d VEGF in ovarian endometriosis (X200).**



Focal VEGF expression in both epithelial and stromal cells.
**Figure 3.1e VEGF in serous borderline ovarian tumour (X200).**



VEGF expression found immediately underlying the epithelium of the tumour.
**Figure 3.1f VEGF in mucinous borderline ovarian tumour (X200).**

Figure 3.2 shows the percentage of each tissue type expressing VEGF. More endometriotic lesions stained positively for VEGF compared to borderline tumours, benign cystadenomas or controls.

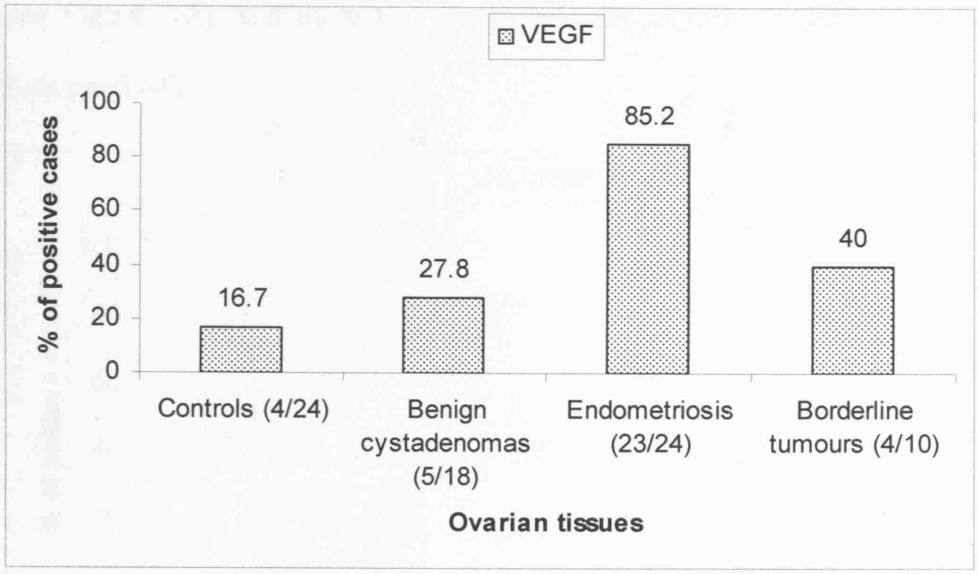



**Figure 3.2 VEGF expression in different ovarian tissues.**

Table 3.4 shows the statistical comparisons of VEGF expression between individual tissues. There is significantly higher VEGF expression in endometriosis compared with the other groups (controls, benign cystadenomas or borderline tumours) with $p<0.05$. This is not surprising as among all these benign conditions, 85.2% of endometriosis express VEGF, thus showing that endometriosis is a highly angiogenic tissue.

| Diagnosis | Controls | Benign cystadenomas | Endometriosis |
|---|---|---|---|
| **Benign cystadenomas** | 0.46 | | |
| **Endometriosis** | **0.0001** | **0.0001** | |
| **Borderline tumours** | 0.20 | 0.68 | **0.01** |

**Table 3.4 P values comparing VEGF expression for each tissue type.**

3.4.1.2 VEGF BETWEEN BENIGN CYSTADENOMA SUBTYPES

When benign cystadenomas were subdivided according to their subtypes, similar percentages of serous and mucinous cystadenomas stained positively for VEGF (see Figure 3.3), and there was no statistically significant differences between them (p=0.68).

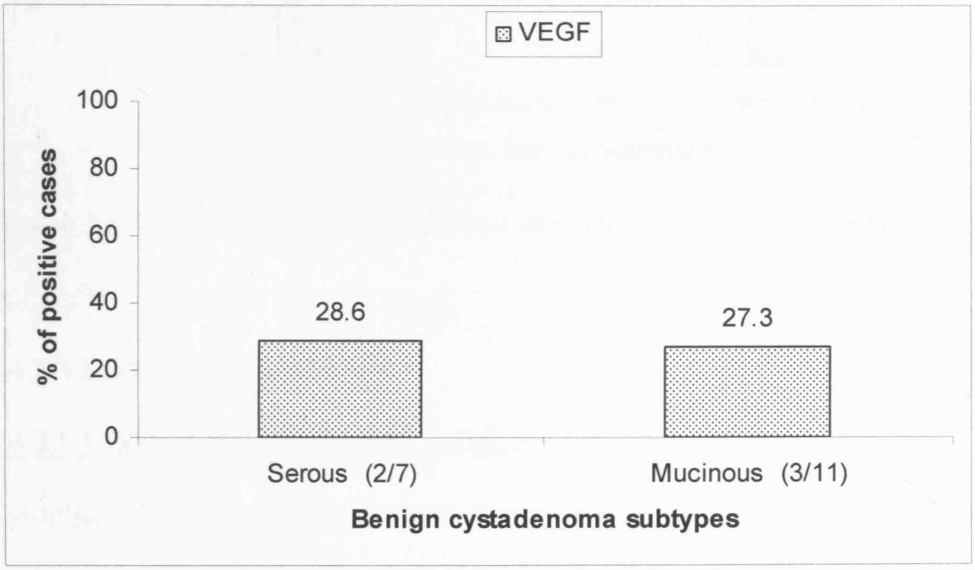



**Figure 3.3 VEGF expression between benign ovarian cystadenoma subtypes.**

3.4.1.3 VEGF BETWEEN BORDERLINE TUMOUR SUBTYPES

Figure 3.4 shows that some cases of serous and endometrioid subtypes were positive for VEGF but the latter was negative in the mucinous subtype, however, no significant difference was found due to the small numbers of samples in this breakdown (p=0.07 for serous versus mucinous and 0.33 for mucinous versus endometrioid).

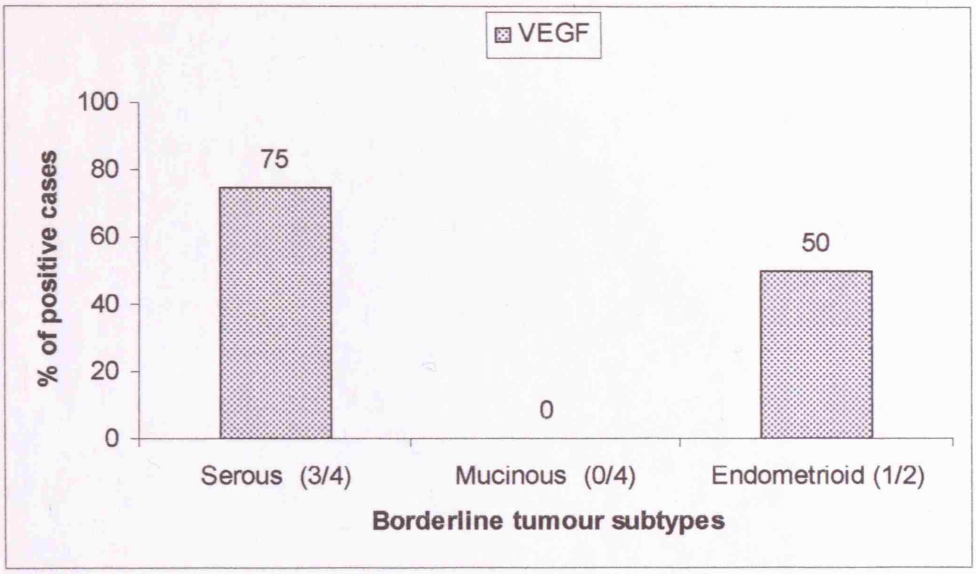
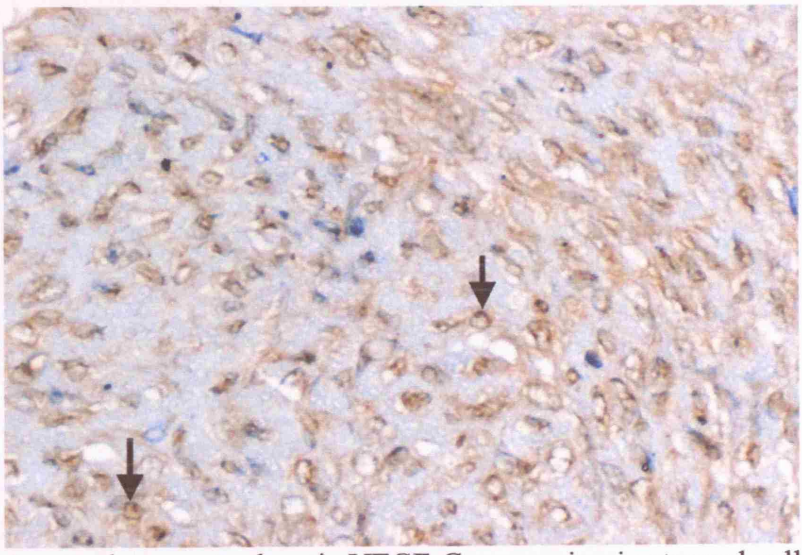



**Figure 3.4 VEGF expression between borderline ovarian tumour subtypes.**

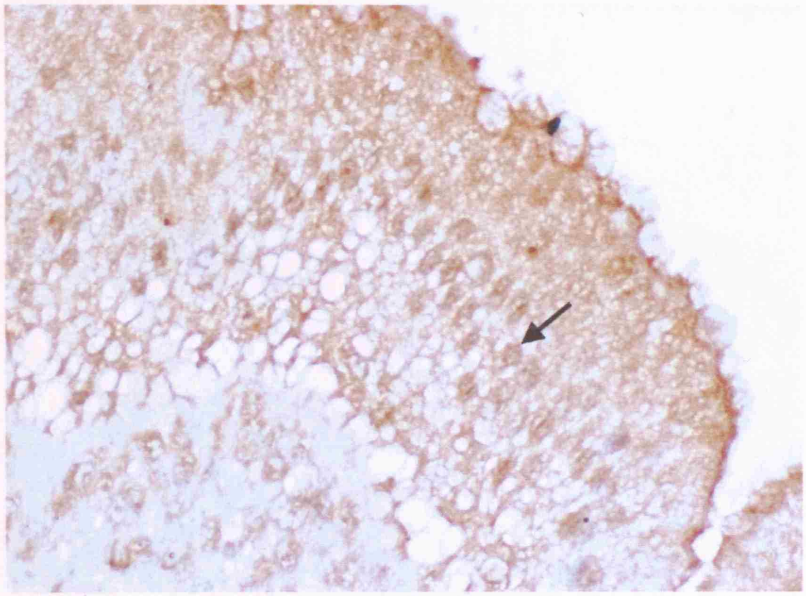
### 3.4.2 VEGF-C EXPRESSION

#### 3.4.2.1 VEGF-C IN OVARIAN TISSUES

Cytoplasmic VEGF-C staining was found in the stromal cells of both benign cystadenomas (Figure 3.5 a-b) and borderline ovarian tumours (Figure 3.5 c-d).

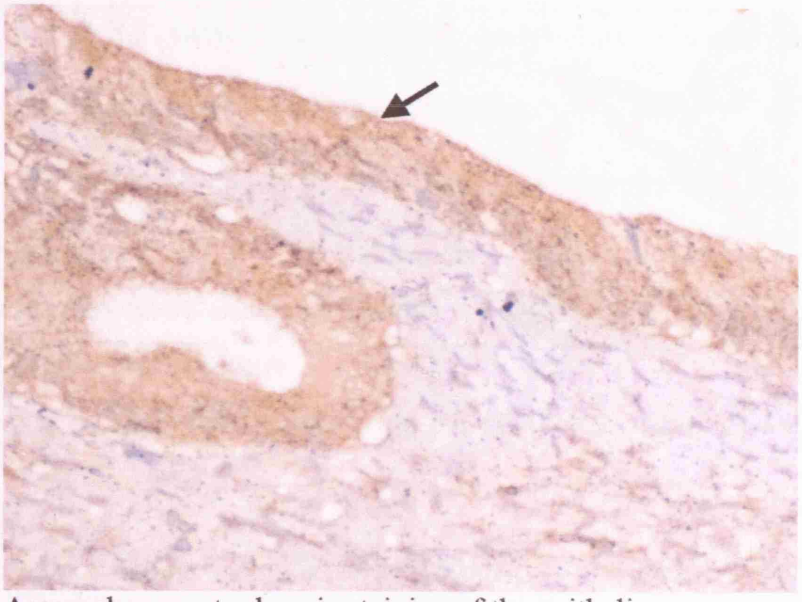


Arrow shows cytoplasmic VEGF-C expression in stromal cells.
**Figure 3.5a VEGF-C in serous ovarian cystadenoma (X400).**



Arrow shows VEGF-C expression immediately underlying the epithelium
**Figure 3.5b VEGF-C in mucinous ovarian cystadenoma (X400).**

Arrow shows cytoplasmic staining mainly on surface of tumour cells.
**Figure 3.5c VEGF-C in serous borderline ovarian tumour (X400).**



Arrow shows cytoplasmic staining of the epithelium.

**Figure 3.5d VEGF-C in mucinous borderline ovarian tumour (X400).**

Figure 3.6 shows a high proportion of benign cystadenomas and borderline tumours expressing VEGF-C but no expression in normal ovarian tissue. Due to scarcity of samples, endometriotic lesions were not stained for VEGF-C.

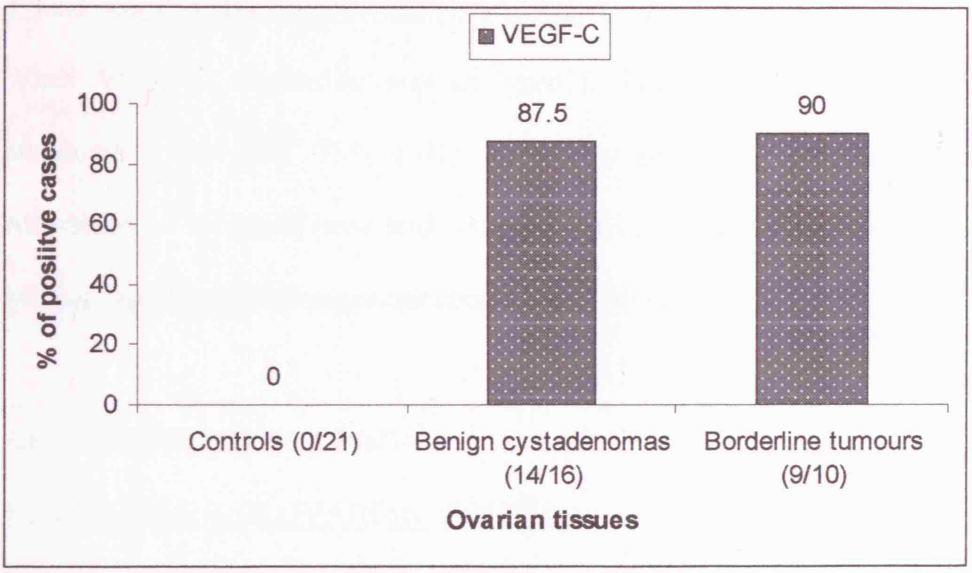



**Figure 3.6 VEGF-C expression in ovarian tissues.**

Table 3.5 shows the statistical analysis of VEGF-C in relation to benign cystadenomas or borderline tumours. A significant difference in VEGF-C expression was found between each of these groups and the normal controls.

| Diagnosis | Controls | Benign cystadenomas |
|---|---|---|
| **Benign cystadenomas** | **0.0001** | |
| **Borderline tumours** | **0.0001** | 0.68 |

**Table 3.5 P values comparing VEGF-C expression for each tissue type.**

3.4.2.2 VEGF-C BETWEEN BENIGN CYSTADENOMA SUBTYPES

Comparisons of VEGF-C expression between benign cystadenoma subtypes showed that 83.3% (5/6) of serous and 90% (9/10) of mucinous cystadenomas stained positively for VEGF-C and this difference was not statistically significant (p=0.63).

3.4.2.3 VEGF-C BETWEEN BORDERLINE TUMOUR SUBTYPES

When VEGF-C expression was analysed in borderline tumours according to histological subtypes, 75% (3/4) of serous and all of the mucinous and endometrioid subtypes (n=4 and n=2 respectively) were positive for VEGF-C, with no significant difference between these subtypes (p=0.44).

### 3.4.3 VEGFR-1 EXPRESSION

3.4.3.1 VEGFR-1 IN OVARIAN TISSUES

Positive VEGFR-1 staining was mainly in the cytoplasm of stromal cells, often appearing like a brown wash staining (Figure 3.7a) and in the epithelial cells of benign ovarian lesions (Figure 3.7 c-e).

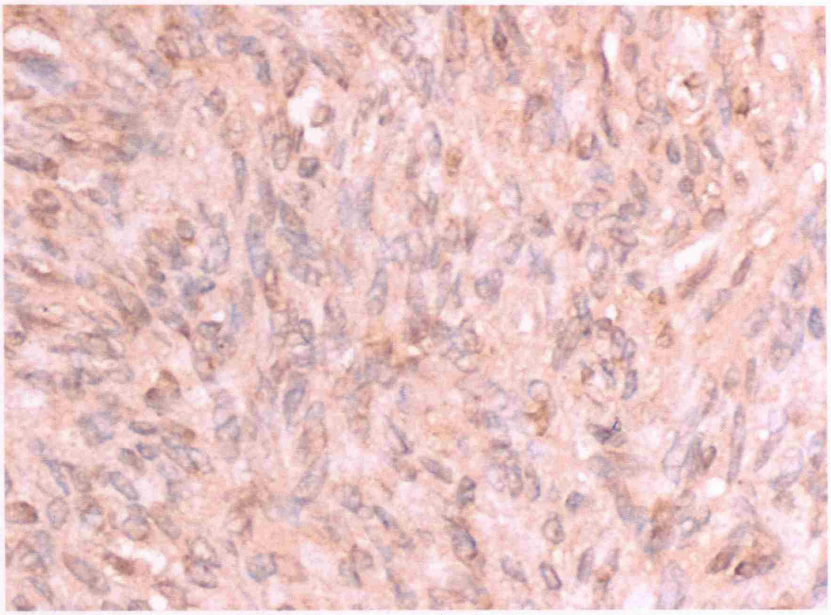
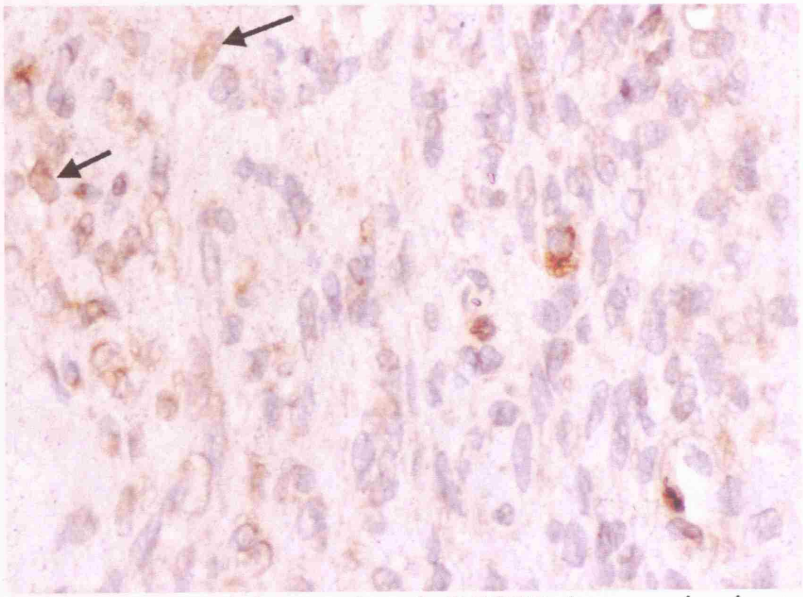
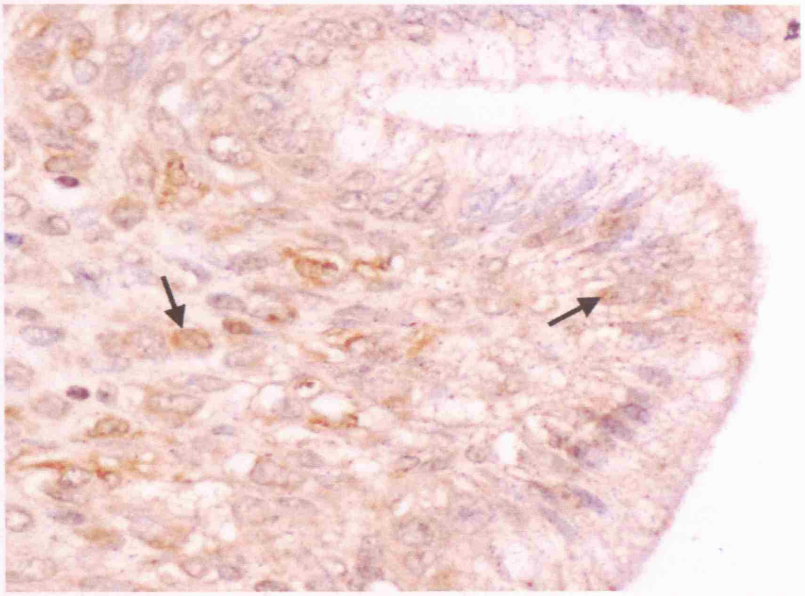


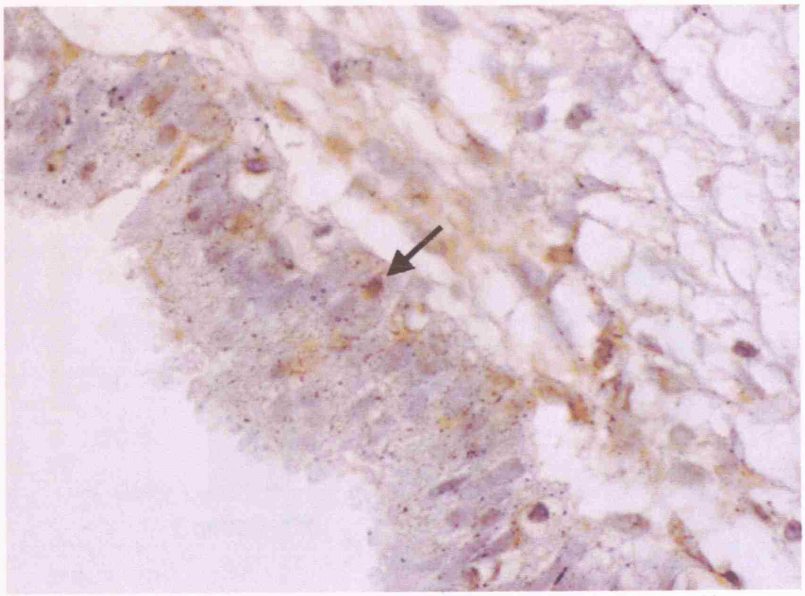
**Figure 3.7a VEGFR-1 in normal ovary, with patchy stromal cells stained (X400).**



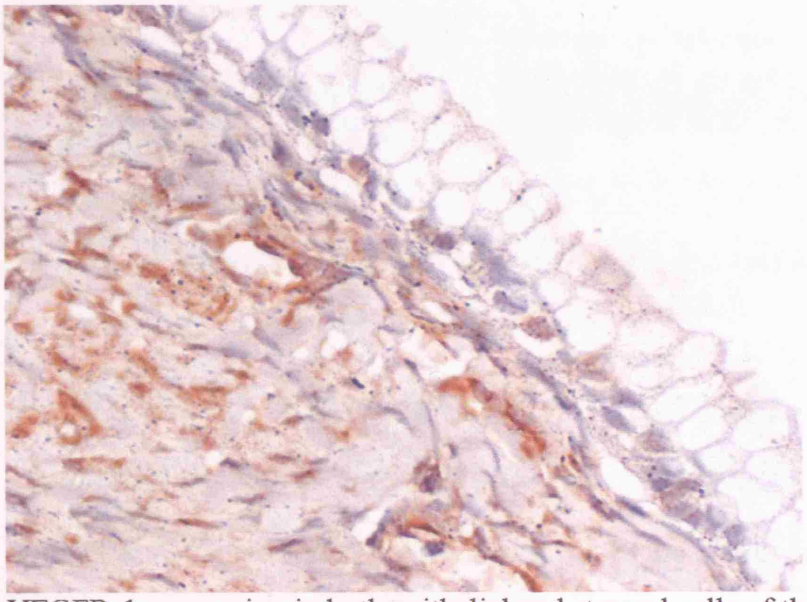
Arrows show mainly cytoplasmic VEGFR-1 expression in stromal cells.
**Figure 3.7b VEGFR-1 in serous ovarian cystadenoma (X400).**



Arrows show cytoplasmic VEGFR-1 staining in both epithelial and stromal cells.
**Figure 3.7c VEGFR-1 in mucinous ovarian cystadenoma (X400).**



Arrow shows VEGFR-1 expression in an epithelial cell.
**Figure 3.7d VEGFR-1 in ovarian endometriosis, staining in both epithelial and stromal cells (X400).**



VEGFR-1 expression in both epithelial and stromal cells of the tumour.
**Figure 3.7e VEGFR-1 in mucinous borderline ovarian tumour (X400).**

Figure 3.8 shows VEGFR-1 expression in ovarian tissues with a high proportion of each ovarian tissue positive for VEGFR-1.

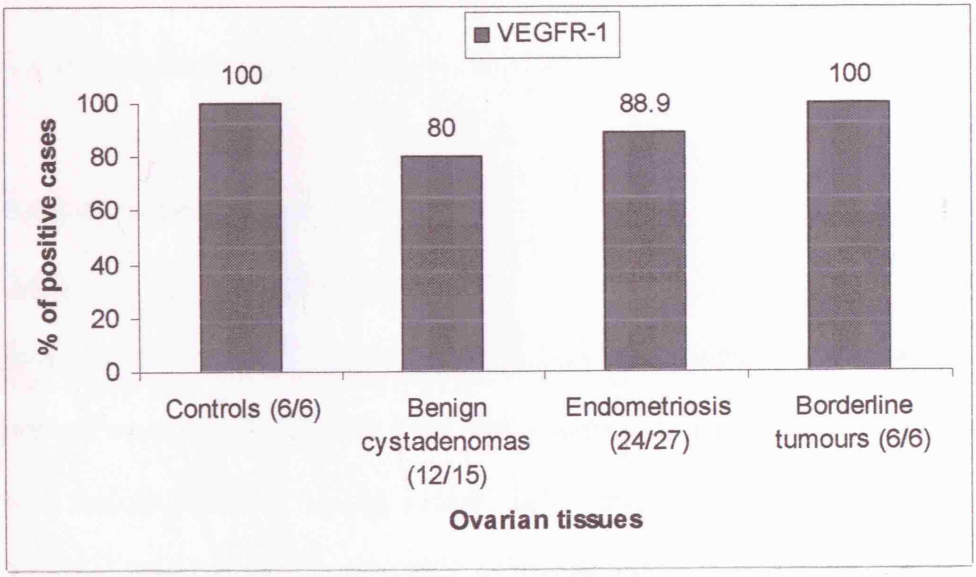



**Figure 3.8 VEGFR-1 expression in ovarian tissues.**

Table 3.6 shows the results of the statistical analysis of the relationship between VEGFR-1 and different tissues. No statistical significant difference was found in VEGFR-1 expression between any groups studied.

| Diagnosis | Controls | Benign cystadenomas | Endometriosis |
|---|---|---|---|
| **Benign cystadenomas** | 0.53 | | |
| **Endometriosis** | 0.54 | 0.65 | |
| **Borderline tumours** | - | 0.53 | 0.54 |

**Table 3.6 P values comparing VEGFR-1 expression for each tissue type.**

3.4.3.2 VEGFR-1 BETWEEN BENIGN CYSTADENOMA SUBTYPES

Analysis of VEGFR-1 expression between benign cystadenoma subtypes showed that 60% (3/5) of serous and 90% (9/10) of mucinous cystadenomas stained positively for VEGFR-1 but this difference is not statistically significant (p=0.24).

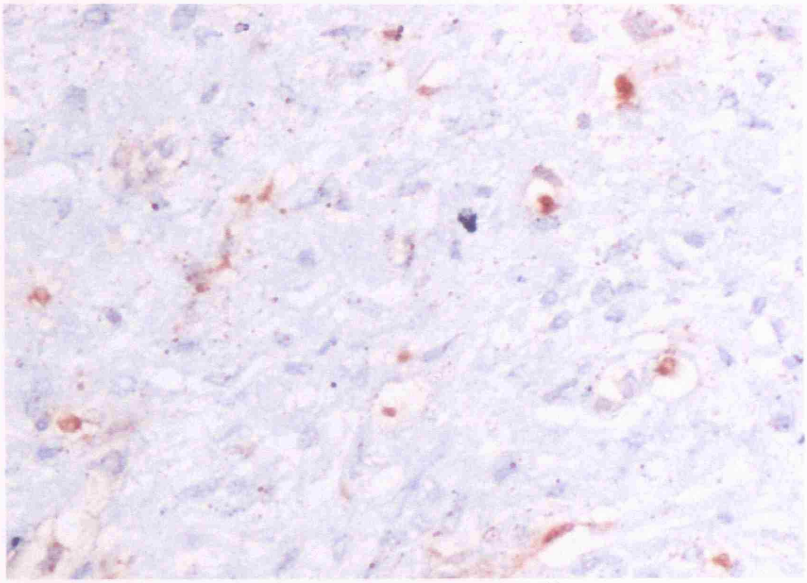
3.4.3.3 VEGFR-1 BETWEEN BORDERLINE TUMOUR SUBTYPES

When VEGF-C expression was analysed in borderline tumours according to histological subtypes, all of the serous, mucinous and endometrioid subtypes (n=3, 2 and 2 respectively) were positive for VEGFR-1.

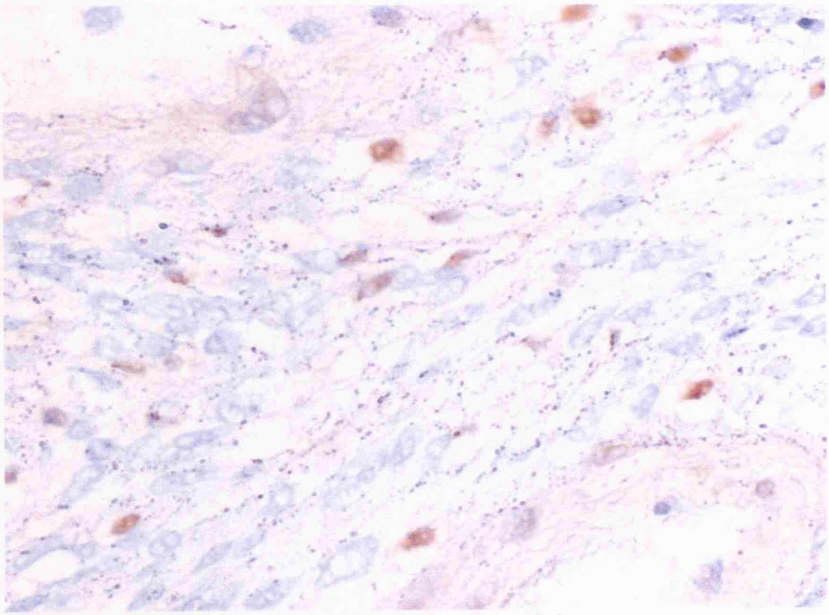
### 3.4.4 TP EXPRESSION

3.4.4.1 TP IN OVARIAN TISSUES

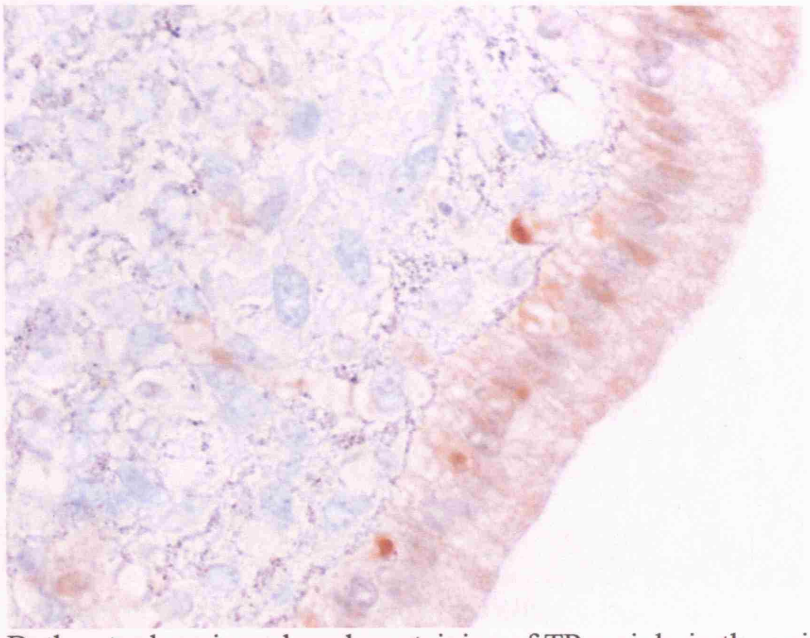
Immunohistochemical staining for TP was both nuclear and cytoplasmic. In normal ovarian cells (Figure 3.9a) and in serous cystadenomas (Figure 3.9b), TP was mainly localised in the stromal cells. In mucinous cystadenomas (Figure 3.9c), endometriosis (Figure 3.9d), and borderline ovarian tumours (Figures 3.9e and f), TP was strongly expressed in epithelial cells of the tumours.



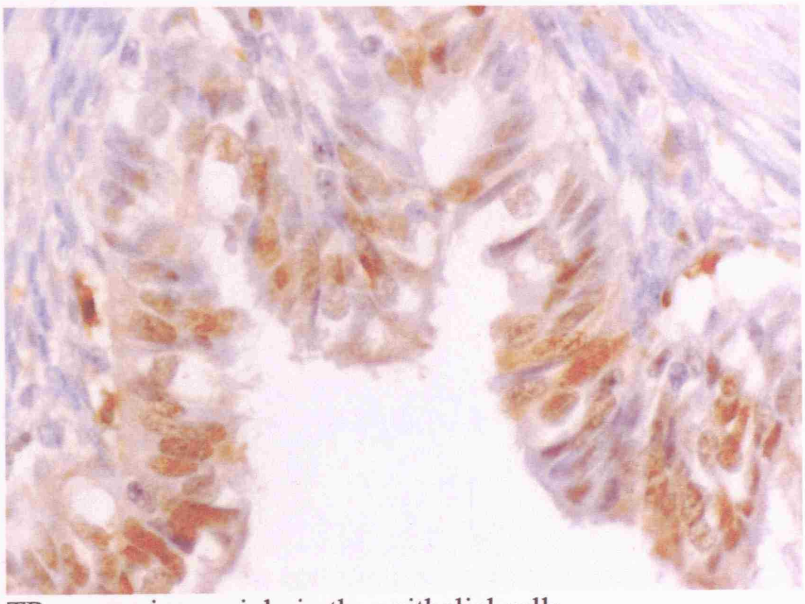
Nuclear TP staining in ovarian stromal cells.
**Figure 3.9a TP in normal ovary (X400).**



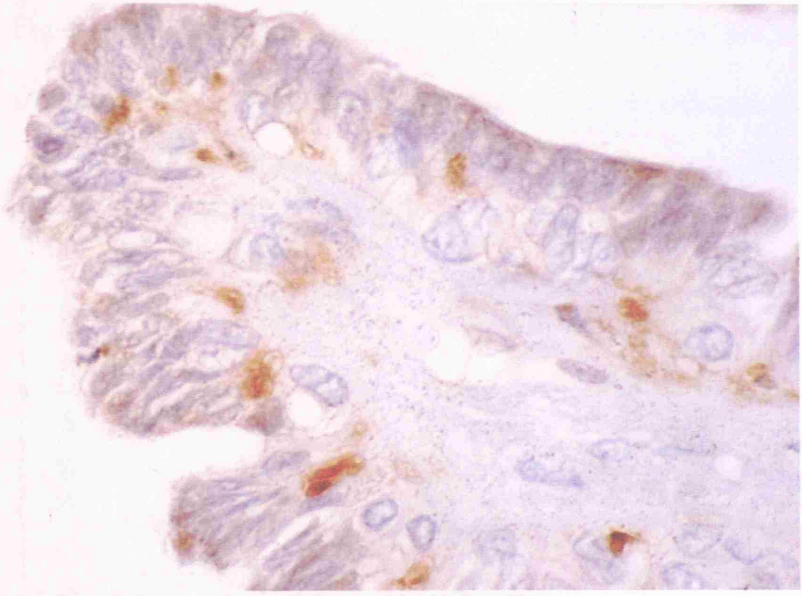
Nuclear TP expression in stromal cells of the tumour.
**Figure 3.9b TP in serous ovarian cystadenoma (X400).**



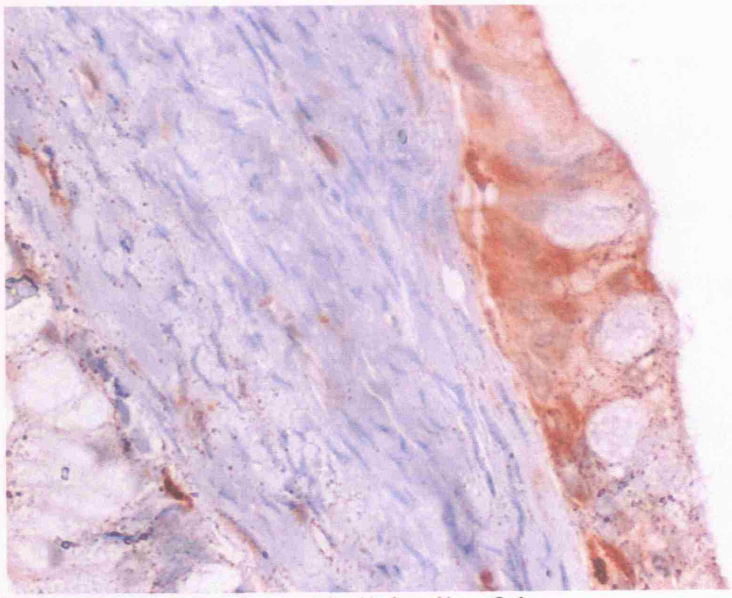
Both cytoplasmic and nuclear staining of TP mainly in the epithelium.
**Figure 3.9c TP in mucinous ovarian cystadenoma (X400).**



TP expression mainly in the epithelial cells.
**Figure 3.9d TP in ovarian endometriosis (X400).**



Nuclear TP staining of the epithelial cells.
**Figure 3.9e TP in serous borderline ovarian tumour (X400).**



TP expression in the epithelial cells of the tumour.
**Figure 3.9f TP in mucinous borderline ovarian tumour (X400).**

Figure 3.10 shows a high proportion of endometriosis (100%) and borderline ovarian tumours (80%) expressing TP compared to the other groups.

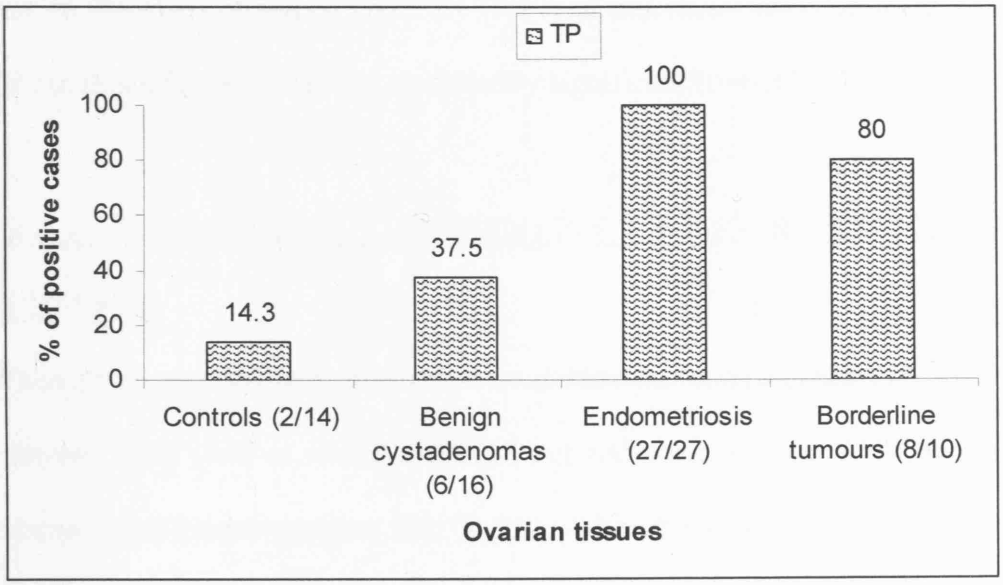



**Figure 3.10 TP expression in ovarian tissues.**

The results of the statistical analysis of TP expression between these ovarian tissues are given in Table 3.7. As can be seen, there is a significant difference between endometriosis and controls and between endometriosis and benign cystadenomas. In addition, a significant difference was also demonstrated in TP expression between borderline tumours and controls.

| **Diagnosis** | **Controls** | **Benign cystadenomas** | **Endometriosis** |
|---|---|---|---|
| **Benign cystadenomas** | 0.23 | | |
| **Endometriosis** | **0.0001** | **0.0001** | |
| **Borderline tumours** | **0.003** | 0.05 | 0.07 |

**Table 3.7 P values comparing TP expression between each tissue type.**

3.4.4.2 TP BETWEEN BENIGN CYSTADENOMA SUBTYPES

A comparison of TP expression between benign cystadenoma subtypes showed that 16.7% (1/6) of serous and 50% (5/10) of mucinous cystadenomas expressed TP but this difference was not statistically significant (p=0.22).

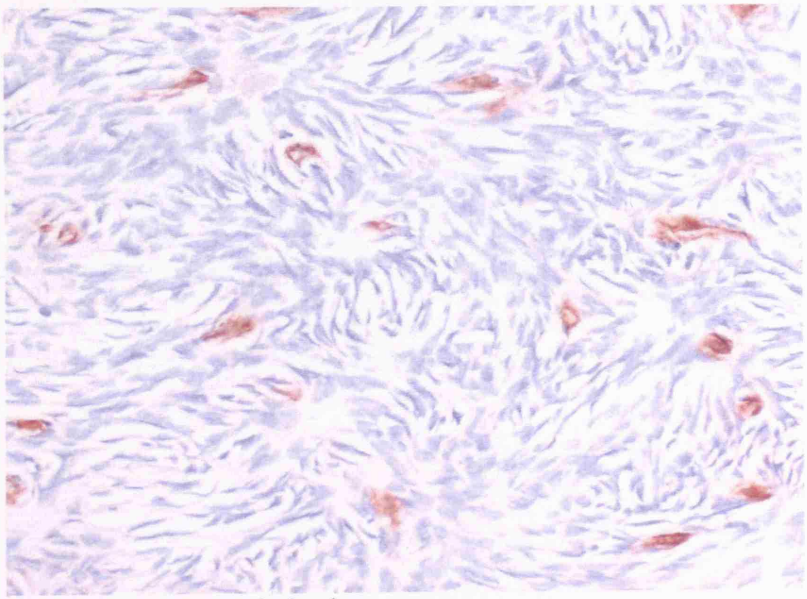
3.4.4.3 TP BETWEEN BORDERLINE TUMOUR HISTOLOGICAL SUBTYPES

When TP expression was analysed in borderline tumours according to histological subtypes, 75% (3/4) of serous, 50% (1/2) of endometrioid and 100% of mucinous subtypes (4/4) were positive for TP, but these differences were not significant (p=0.34).

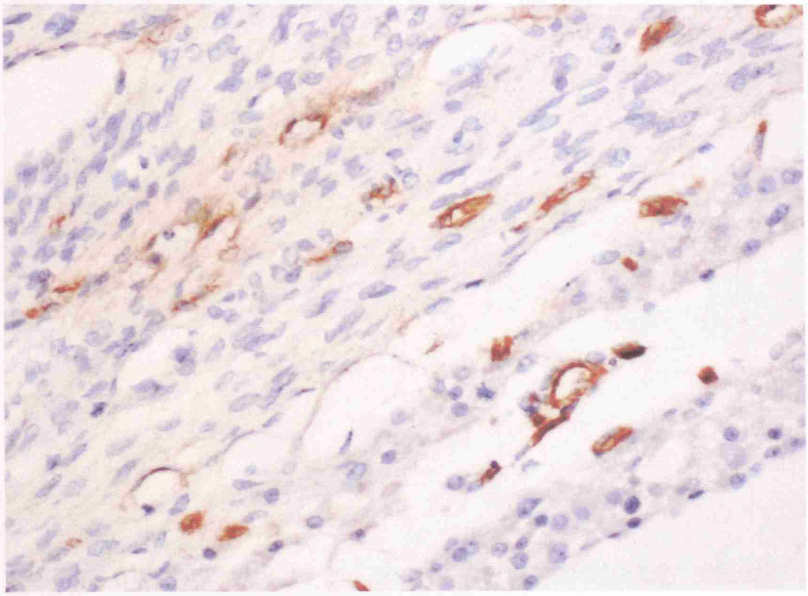
### 3.4.5 MICROVESSEL DENSITY (MVD)

3.4.5.1 MVD IN OVARIAN TISSUES

VWF immunostaining was generally found in the endothelial cells lining the microvessels found in the stromal area.

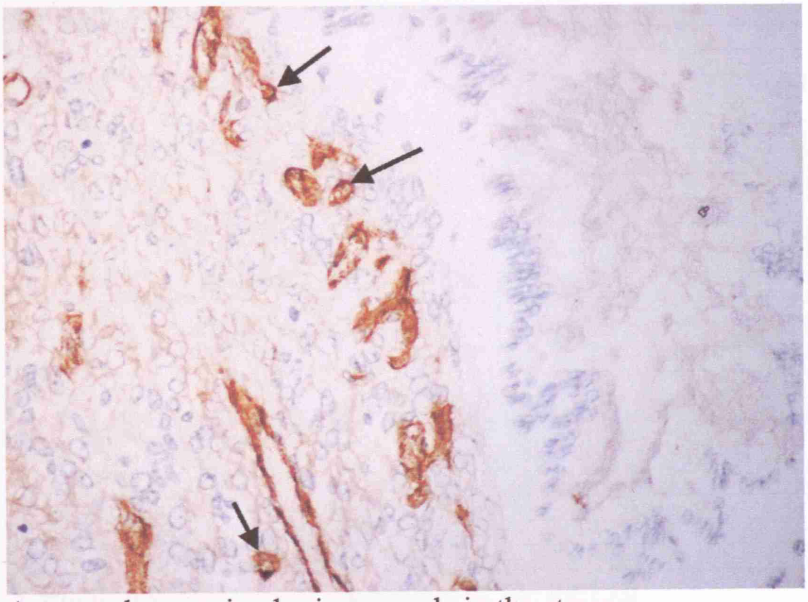


Stained microvessels in the stroma.
**Figure 3.11a MVD in normal ovary (X200).**



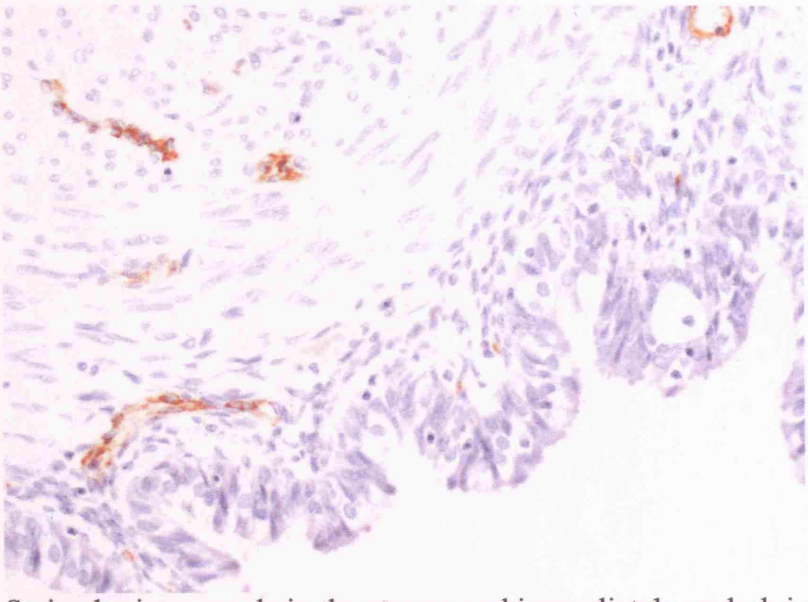
Stained microvessels in the stroma.

**Figure 3.11b MVD in serous ovarian cystadenoma (X200).**

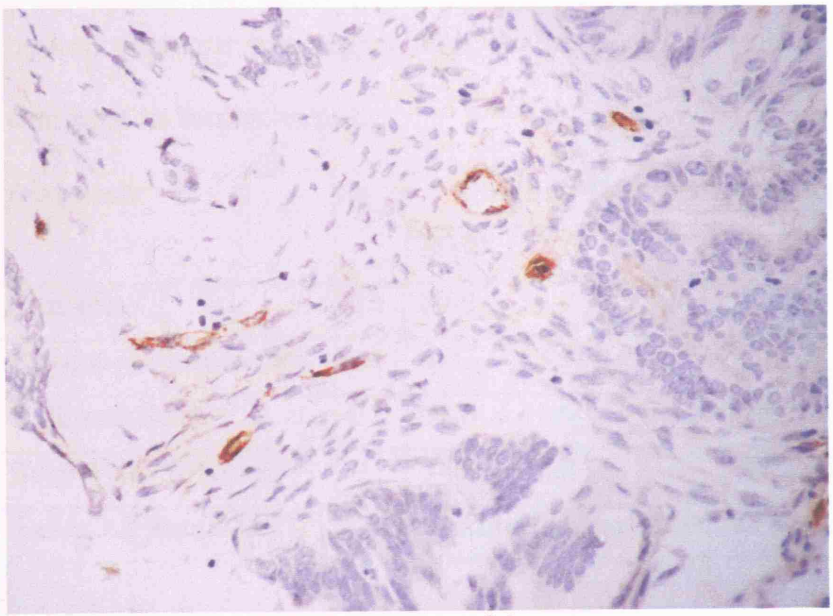


Arrows show stained microvessels in the stroma.

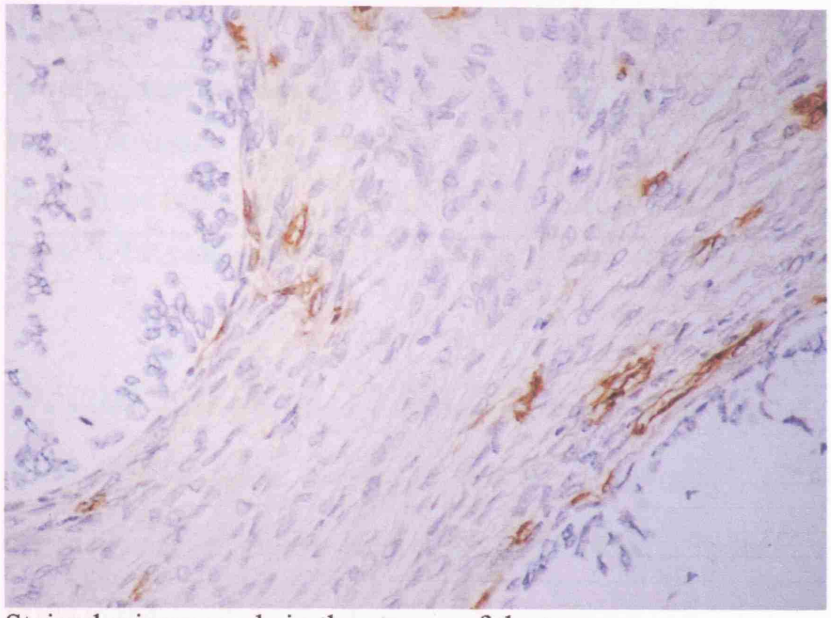
**Figure 3.11c MVD in mucinous ovarian cystadenoma (X200).**



Stained microvessels in the stroma and immediately underlying the epithelium.
**Figure 3.11d MVD in ovarian endometriosis (X200).**



Stained microvessels in the stroma of the tumour.
**Figure 3.11e MVD in serous borderline ovarian tumour (X200).**



Stained microvessels in the stroma of the tumour.

**Figure 3.11f MVD in mucinous borderline ovarian tumour (X200).**

Table 3.8 shows the mean HVD and AVD values classified according to different tissues, demonstrating higher MVD values in the normal ovary control group compared to benign lesions.

| **Diagnosis** | **n** | **Mean MVD ± SD values per mm$^2$** | |
|---|---|---|---|
| | | **HVD** | **AVD** |
| **Controls** | 23 | 80.8 ± 19.9 | 67.1 ± 16.1 |
| **Benign cystadenomas** | 15 | 68.2 ± 18.3 | 60.1 ± 16.5 |
| **Endometriosis** | 27 | 56.0 ± 31.6 | 44.7 ± 23.8 |
| **Borderline tumours** | 9 | 54.8 ± 22.0 | 47.2 ± 21.5 |

**Table 3.8 Mean MVD values at magnification X200 for the ovarian tissues.**

Statistical analysis of MVD in relation to different tissues shows significantly smaller MVD values in controls compared with endometriosis and borderline tumours (Table 3.9).

| Diagnosis | Controls | Benign cystadenomas | Endometriosis |
|---|---|---|---|
| **Benign cystadenomas** | 0.06 (0.20) | | |
| **Endometriosis** | **0.002 (0.0001)** | 0.18 (0.03) | |
| **Borderline tumours** | **0.003 (0.008)** | 0.12 (0.12) | 0.92 (0.78) |

**Table 3.9 P values for HVD (AVD) in relation to each ovarian tissue type.**

3.4.5.2 MVD BETWEEN HISTOLOGICAL SUBTYPES

When MVD was analysed between histological parameters in benign cystadenomas and borderline ovarian tumours, no differences were found between MVD in the different histological subtypes (see Table 3.10).

| Diagnosis | Mean ± SD per $mm^2$ | | p value |
|---|---|---|---|
| | HVD | AVD | HVD, AVD |
| **Cystadenomas** | | | |
| **Serous (n=5)** | 60.8 ± 23.1 | 56.1 ± 22.0 | |
| **Mucinous (n=10)** | 71.6 ± 15.4 | 62.1 ± 13.9 | 0.16, 0.24 (serous vs mucinous) |
| **Borderline tumours -subtypes** | | | |
| **Serous (n=4)** | 58.4 ± 27.6 | 50.6 ± 24.0 | 0.33, 0.26 (serous vs mucinous) |
| **Mucinous (n=4)** | 43.5 ± 5.9 | 35.2 ± 6.6 | 0.08, 0.08 (mucinous vs endometrioid) |
| **Endometrioid (n=2)** | 85.05 | 81.95 | 0.45, 0.33 (serous vs endometrioid) |

**Table 3.10 Mean MVD values between histological subtypes (at X200).**

### 3.4.6 PROTEIN EXPRESSION AND MVD

Table 3.11 shows that MVD values tend to be higher in benign neoplasms expressing the angiogenic protein analysed compared to their negative corresponding counterparts. In addition, a statistically significant difference in MVD was found between VEGF-positive borderline tumours compared to VEGF-negative borderline tumours (0.001).

| | Number | Mean ± SDs values of | | p value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| **Cystadenomas** | | | | | |
| VEGF negative | **10** | 61.3 ± 14.3 | 52.9 ± 10.2 | | |
| VEGF positive | 5 | 82.1 ± 18.7 | 74.3 ± 18.3 | 0.069 | 0.057 |
| VEGF-C negative | 2 | 66.1 | 55.4 | | |
| VEGF-C positive | 14 | 68.4 ± 18.9 | 60.4 ± 17.0 | 0.912 | 0.777 |
| VEGFR-1 negative | 4 | 58.4 ±17.6 | 50.9 ± 16.1 | | |
| VEGFR-1 positive | 11 | 71.8 ± 17.9 | 63.4 ± 16.0 | 0.247 | 0.239 |
| TP negative | 9 | 65.7 ± 16.3 | 58.7 ± 15.0 | | |
| TP positive | 6 | 72.0 ± 21.9 | 62.3 ± 19.4 | 0.563 | 0.710 |
| **Endometriosis** | | | | | |
| VEGF negative | 4 | 54.7 ± 21.6 | 47.6 ± 19.3 | | |
| VEGF positive | 23 | 56.2 ± 33.4 | 44.2 ± 24.8 | 0.932 | 0.798 |
| VEGFR-1 negative | 4 | 35.1 ± 23.1 | 27.0 ± 17.5 | | |
| VEGFR-1 positive | 23 | 59.6 ± 31.8 | 47.8 ± 23.6 | 0.156 | 0.108 |
| TP negative | 8 | 49.4 ± 15.6 | 40.7 ± 12.1 | | |
| TP positive | 19 | 58.7 ± 36.3 | 46.4 ± 27.3 | 0.448 | 0.577 |
| **Borderline tumours** | | | | | |
| VEGF negative | 5 | 38.3 ± 12.7 | 31.1 ± 10.7 | | |
| VEGF positive | 4 | 75.3 ± 8.0 | 67.4 ± 10.1 | **0.001** | **0.001** |
| VEGF-C negative | 1 | 78.3 | 66.5 | | |
| VEGF-C positive | 8 | 51.8 ± 21.5 | 44.8 ± 21.6 | 0.284 | 0.375 |
| VEGFR-1 negative | 0 | | | | |
| VEGFR-1 positive | 10 | 57.4 ± 24.9 | 56.2 ± 24.3 | ua | ua |
| TP negative | 1 | 78.3 | 66.5 | | |
| TP positive | 8 | 51.8 ± 21.6 | 44.8 ± 21.6 | 0.284 | 0.375 |

Ua:unaccountable

**Table 3.11 MVD between positive and negative cases for each angiogenic protein.**

Again, owing to the small number of cystadenoma sybtypes and borderline tumour subtypes and grades, comparisons of each protein expression with MVD

between these histological parameters were not possible to perform as the number of samples did not meet the criteria in order to perform statistical analysis.

## 3.5 DISCUSSION

### 3.5.1 VEGF

#### 3.5.1.1 VEGF IN BENIGN CYSTADENOMAS

Absence or weak VEGF immunostaining has been demonstrated in benign ovarian cystadenomas by a number of authors (Yamamoto et al, 1997; Orre and Rogers, 1999; Shen et al; 2000; Brustmann and Naude, 2002, Brustmann, 2004; Chen et al, 2004; Li et al, 2005). Using a polyclonal antibody for VEGF, Shen et al found 6% (1/17) ovarian cystadenomas had low expressions for VEGF and none had high expression (Shen et al, 2000) while another study found 51% (21/41) of cystadenomas VEGF-positive (Brustmann, 2004). In the present study, the use of a monoclonal antibody, use of protease digestion and the robust scoring system showed that 27.8% (5/18) of benign tumours compared to 16.7% (4/24) of normal ovaries respectively, stained positively for VEGF. These discrepancies may therefore depend on differences of methodology (for example, different antigen retrieval steps) or specificity of antibodies used in each experiment. However, the greater numbers of benign cystadenomas expressing VEGF compared to controls suggests that the process of neovascularisation is slightly greater in the neoplastic process than in normal ovarian tissue.

In addition, from this study, VEGF protein expression was similarly positive in both serous and mucinous cystadenoma subtypes. A focal pattern of VEGF expression and of weak staining intensity was also reported in (6/10) serous cystadenomas by Brustmann and Naude; however, no other subtype was

mentioned in their study (Brustmann and Naude, 2002). These results suggest that VEGF plays an important role in the development of both serous and mucinous ovarian neoplasms.

### 3.5.1.2 VEGF IN ENDOMETRIOSIS

Several authors have demonstrated the presence of VEGF in human endometrium and that it may be important in both physiological and pathological angiogenesis (Charnock-Jones et al, 1993; Smith, 1996). Others have demonstrated higher peritoneal concentrations of VEGF in women with moderate to severe endometriosis than in women without the disease (McLaren et al, 1996b; Shifren et al, 1996). A study on twenty-eight patients with ovarian endometriosis, showed diffuse expression of VEGF in epithelial cells which was associated with cyst diameters greater than 5.4 cm, and high VEGF expression in capsular fibroblasts associated with bilateral cysts (Goteri et al, 2004). Another study showed that VEGF levels in serum of 30 women with endometriosis were higher than those in women with serous cystadenomas (Artini et al, 2005).

In the present study, 85.2% of the ovaries from 24 women with endometriosis expressed VEGF compared to 16.7% of the control group, and this difference is highly statistically significant ($p=0.0001$). These high levels of VEGF suggest that VEGF-induced angiogenesis may be a critical factor for the pathophysiology of endometriosis.

### 3.5.1.3 VEGF IN BORDERLINE TUMOURS

Numerous studies have demonstrated that the expression of VEGF can predict an increased risk of metastatic disease and reduced overall survival by stimulating angiogenesis in ovarian carcinomas and other carcinomas such as non-small cell lung cancer and gastric cancers (Gadduchi et al, 1999; Kim et al, 2003; Shi et al, 2003; Song et al, 2002). Recently, in a large study of 69 borderline tumours, Li et al showed that VEGF protein was positive in 59.4% of borderline tumours and that VEGF expression rate was higher than in benign tumours and lower than in ovarian carcinomas, and there was a significant difference between borderline tumours and benign ovarian tumours ($p<0.05$), and carcinoma ($p<0.01$; Li et al, 2005).

In this study, on a much smaller number of borderline tumours analysed (n=10), 40% of the borderline tumours stained positively for VEGF expression compared to normal ovary (16.7%, $p>0.05$) and endometriotic lesions (85.2%, $p=0.01$). There was no correlation between VEGF expression and histological subtypes in borderline tumours, possibly due to the small number of different subtypes studied. Abu-Jawdeh et al (1996) demonstrated variable VEGF mRNA expression in serous borderline tumours but no definite expression in mucinous borderline tumours. Such data suggest that VEGF is important in borderline lesions; however, little is known about the mechanisms of angiogenesis taking place in these borderline tumour subtypes.

### 3.5.2 VEGF-C

#### 3.5.2.1 VEGF-C IN BENIGN CYSTADENOMAS

A study by Yokoyama et al (2003) on benign cystadenomas showed that 40% (8/20) had weak VEGF-C staining and the remaining 12 were negative for VEGF-C. Another study by Nishida et al, (2004) reported 9.1% (2/22) of the benign cystadenomas studied were VEGF-C positive. Data from this study, on approximately the same number of benign ovarian samples (n = 21) and the use of the same antibody (polyclonal anti-VEGF-C) but from a different supplier showed 87.5% (14/16) of benign cystadenomas to express VEGF-C compared to 0% (0/21) of normal ovaries. These discrepancies in results may be explained by the difference in methodology used. In this study, pressure cooking was used for the antigen retrieval step whereas Yokoyama et al used microwaving. As a result, these conflicting data should be interpreted with caution. However, all these studies showed that VEGF-C may have an influential role in benign conditions rather than in normal physiological events. Furthermore in this current study, the benign cases were subdivided according to their histological subtypes but no difference in VEGF-C expression was found between them. It is therefore unlikely that specific histological subtypes of benign ovarian tumours have different lymphangiogenic pathways.

#### 3.5.2.2 VEGF-C IN BORDERLINE TUMOURS

Studies by Yokoyama et al (2003) reported that 18% (2/11) of borderline tumours showed moderate staining for VEGF-C, and Nishida et al (2004) that 40% of the 10 borderline tumours expressed VEGF-C and there was a statistically significant difference between benign cystadenomas and borderline tumours ($p< 0.04$).

The current study also analysed 10 borderline tumours but the results were somewhat surprising and need interpreting with caution. Ninety percent of borderline tumours were found positive for VEGF-C, with a significant difference when compared to controls (p=0.0001) but not significant when compared to benign cystadenomas. It is possible that these differences may be explained by the use of pressure cooking as an antigen retrieval step in this study. Pressure cooking is known to be a rather strong and 'rough' way of exposing the antigens to the antibodies on tissues, and this may result in higher levels of expression of VEGF-C that may remain undetected in immunostaining method without pressure cooking. However, since all groups in this series were treated the same way, similar levels of VEGF-C expression in both benign cystadenomas and borderline tumours suggest that VEGF-C is important in the lymphangiogenic process occurring in these two benign neoplasms.

### 3.5.3 VEGFR-1

#### 3.5.3.1 VEGFR-1 IN BENIGN CYSTADENOMAS

To my knowledge, few IHC studies of VEGFR-1 in ovarian tissues are present in the literature. Goede et al (1998) found that both VEGFR-1 and VEGFR-2 receptors were localised in endothelial cells of the corpus luteum. A further study revealed that VEGFR-1 expression was detected in granulosa and thecal cells of pre-ovulatory follicles as well as in endothelial cells in 28 patients with regular menstrual cycles (Otani et al, 1999). Most recently, immunohistochemistry on 15 corpora lutea showed that both VEGFR-1 and VEGFR-2 receptors are expressed in early and mid luteal phase compared to late luteal phase (Endo et al, 2001).

In this current study, all 6 normal human ovaries and 16 of the 20 (80%) benign cystadenomas expressed VEGFR-1. These findings are in accord with a recent study, in which Chen et al (2004) reported similar immunohistochemical expressions of VEGFR-1 and VEGFR-2 in both benign cystadenomas and normal ovaries. These findings confirm that angiogenesis in normal and benign ovarian lesions is promoted through these receptors on vascular endothelial cells.

3.5.3.2 VEGFR-1 IN ENDOMETRIOSIS

VEGFR-1 and VEGFR-2 are tyrosine kinase receptors expressed predominantly on endothelial cells (Ferrara and Henzel, 1989; Jakeman et al, 1992). They are also found on non-endothelial cells such as peripheral blood monocytes which are the cellular precursors of macrophages (Shen et al, 1993), malignant melanoma cell lines (Gitay-Goren et al, 1993), ovarian carcinoma tumour cells (Boocock et al, 1995) and trophoblasts (Charnock-Jones et al, 1994). In the literature, McLaren et al (McLaren et al, 1996) using RT-PCR reported the presence of both types of VEGF receptors on peritoneal fluid macrophages in endometriosis. The co-expression of VEGF and its receptors raises the possibility of autocrine stimulation and of therapeutic strategies targeting this receptor-ligand interaction. Recently, Artini et al found higher VEGFR-1 concentration in serum of patients with ovarian endometriosis than in patients with benign serous cystadenomas (Artini et al, 2005).

Data from this study showed that VEGFR-1 was immunopositive in 88.9 % of women with endometriosis and 100% of controls (6 normal ovaries). These findings are in accordance with those of McLaren et al and Artini et al (McLaren et al, 1996; Artini et al, 2005) who suggested a possible link between

endometriotic angiogenesis and VEGF. Consequently, the VEGF pathway, via its receptors, may be a critical aspect of the pathophysiology of endometriosis and may indicate a clinical approach to its medical management, via the use of anti-angiogenic treatments.

#### 3.5.3.3 VEGFR-1 IN BORDERLINE TUMOURS

In angiogenesis and lymphangiogenesis, VEGFR-1 and VEGFR-2 are known to act as receptors for VEGF and VEGF-C (Veikkola et al, 2000; Mandriota et al, 2001; Rafii et al, 2003). Using *in situ* hybridisation, Abu-Jawdeh et al, reported that all 6 borderline tumours analysed were positive for both VEGFR-1 and VEGFR-2 (Abu-Jawdeh et al, 1996).

This current study also showed strong VEGFR-1 expression in all borderline tumours (n=10) which reflects the importance of these receptors to which VEGF will bind, essential for the angiogenic events taking place in borderline tumours to promote tumour proliferation. In addition, the co-expression of VEGF and VEGFR-1 in the epithelial component of borderline tumours suggests a potential autocrine stimulation and the stromal expression of VEGF acting on epithelial VEGFR-1 suggests an additional paracrine stimulation, both of which may indicate pre-malignant events.

### 3.5.4 TP

#### 3.5.4.1 TP IN BENIGN CYSTADENOMAS

Recently, Miszczak-Zaborska et al (2004) used a spectrophotometric method and showed that TP activity was lower in the cytosol of normal ovaries, benign tumours and borderline tumours than in malignant tumours. In this study, 37.5%

of benign ovarian cystadenomas compared to 14.3% of normal ovaries were positive for TP expression, although this was not statistically significant (p=0.23). These various patterns but rather low TP protein expression in normal and benign tissues support several possible roles played by TP in both normal physiological processes as well as in benign conditions.

No study in the literature, except this one, has compared TP protein expression between benign cystadenoma subtypes. In this study, TP expression was found higher in mucinous compared to serous ovarian cystadenomas. This finding although not statistically significant, may reflect higher angiogenic activity regulated by TP in mucinous subtype compared to other ovarian cystadenomas and may explain why the mucinous subtype may represent an intermediate stage in the stepwise sequence progression to ovarian cancer.

### 3.5.4.2 TP IN ENDOMETRIOSIS

Studies carried out by Fujimoto et al (1999) found that TP was expressed in the epithelial cells of ovarian endometriomas and in interstitial cells of the subepithelial area (Fujimoto et al; 1999). The same group also reported the expression of TP in endometriotic endometrium during the menstrual cycle (Fujimoto et al, 1996; 1999) and suggested that TP contributes to the growth of endometriomas via subepithelial angiogenesis independently of the sex steroidal milieu.

TP expression in this study was over-expressed in all 27 women with ovarian endometriosis compared to controls (14.3%) or benign cystadenomas (37.5%); this difference was statistically significant (p= 0.0001 and 0.0001 respectively), suggesting that different mechanisms including TP-angiogenic related pathway or

VEGF and VEGFR-1 pathway may both contribute to an acceleration of angiogenesis in the pathogenesis of ovarian endometriosis.

3.5.4.3 TP IN BORDERLINE TUMOURS

To my knowledge, few studies have analysed TP protein expression in borderline ovarian tumours. Miszczak-Zaborska et al (2004) using an enzyme assay reported a higher mean TP activity in serum specimens from patients with borderline tumours compared to women with normal ovaries. These data are in accord with the IHC results of my study showing TP expression in a higher proportion of borderline tumours (80%) compared to controls (14.3%) and this difference was statistically significant (p=0.003). These findings clearly indicate that TP expression or TP activity is increased along the neoplastic transformation of these tumours, probably favouring malignant transformation.

**3.5.5 MVD**

3.5.5.1 MVD IN BENIGN CYSTADENOMAS

It is now well established that von Willebrand factor (vWF), a glycoprotein produced only by endothelial cells, can be used to identify vessels in tissue sections. Recent work using vWF in paraffin wax-embedded sections of ovaries showed highest MVD values in functional cysts and similar values in non-functional and benign tumours (Amis et al, 2005). In this study, the tendency for higher MVD values in the control group is partly due to the presence of pre-menopausal women included in the controls (16 pre- and 8 post-menopausal women). Hence the higher MVD values agree with the findings of Amis et al study (2005) and reflect the high vascularity in normal functional cysts.

Mucinous cystadenomas demonstrated a higher MVD than serous cystadenomas, suggesting a difference in the genetic profile and the biology of these morphological subtypes of ovarian tumours. These findings are also related to the high VEGF and TP expression observed in mucinous cystadenomas when compared with the serous subtype, thus suggesting that different angiogenic mechanisms are taking place in these different subtypes.

3.5.5.2 MVD IN ENDOMETRIOSIS

In this study, AVD and HVD in endometriosis was significantly lower (44.7 ± 23.8 and 56.0 ± 31.6 respectively) than those in controls (p=0.0001 and 0.002 respectively). This decrease in vascularity contradicts with the high levels of VEGF and TP found in these endometriotic lesions. A possible explanation may be due to the use of vWF which highlights both pre-existing and newly formed blood vessels. Endoglin, a specific marker for newly formed vessels is therefore more appropriate for measuring angiogenesis and for clarifying this contradictory result.

3.5.5.3 MVD IN BORDERLINE TUMOURS

In the study by Shen et al (2000) including 13 borderline and 17 benign ovarian cystadenomas, the authors reported similar mean MVD values between these tumours. Results from this study showed lower MVD values in borderline tumours than in benign cystadenomas. There was no correlation between MVD and histological parameters of these borderline tumours. Again, the use of endoglin a more specific marker for tumour neovascularisation is needed to clarify these discrepancies in results.

### 3.5.6 PROTEIN EXPRESSION AND MVD

#### 3.5.6.1 IN BENIGN CYSTADENOMAS AND ENDOMETRIOSIS

In this study, MVD in both benign cystadenomas and endometriotic lesions positive for VEGF, VEGF-C, VEGFR-1 or TP was higher than in their corresponding negative benign tumours, though this was not significant. This finding clearly indicates that any change related to the levels of the angiogenic factors, happening in these benign cysts and endometriotic lesions, is accompanied by an increase in microvessels necessary for the growth and maturation of these cysts.

#### 3.5.6.2 IN BORDERLINE TUMOURS

Borderline tumours positive for VEGF showed a significantly higher MVD values than in VEGF negative borderline tumours ($p= 0.01$), however no differences were found when compared MVD in cases positive and negative for other angiogenic factors. These observations are in accord with the work of Nakanishi et al, in that the MVD of VEGF rich tumours was significantly higher than that of VEGF poor tumours (Nakanishi et al, 1997). The increase in MVD related to VEGF positivity in borderline tumours suggests that increased VEGF expression in borderline tumours is accompanied by an increase in blood vessel vasculature, thus enhanced angiogenesis that may be responsible for the growth activity of these tumour cells.

## 3.6 CONCLUDING RESULTS

In summary, the findings from this chapter are as follows:

1. Both VEGF and TP are increased significantly in the formation of endometriotic and borderline lesions. This shows that these two angiogenic stimulators are important in the formation of new blood supply in these pre-malignant ovarian lesions via separate molecular pathways.
2. The increase in VEGF-C levels in serous and mucinous subtypes of benign cystadenomas and borderline ovarian tumours suggest that lymphangiogenesis is involved in these benign tumours; however, nothing is known about how it works
3. The high levels of VEGF expressed in the borderline tumours are specific to serous and endometrioid subtypes, reflecting specific angiogenic pathways involved in these lesions compared to mucinous subtypes.
4. As VEGF and its receptor are co-localised in all the ovarian lesions, it seems that it is highly likely that an autocrine mechanism is making a major contribution to angiogenesis in the pre-malignant ovarian lesions.

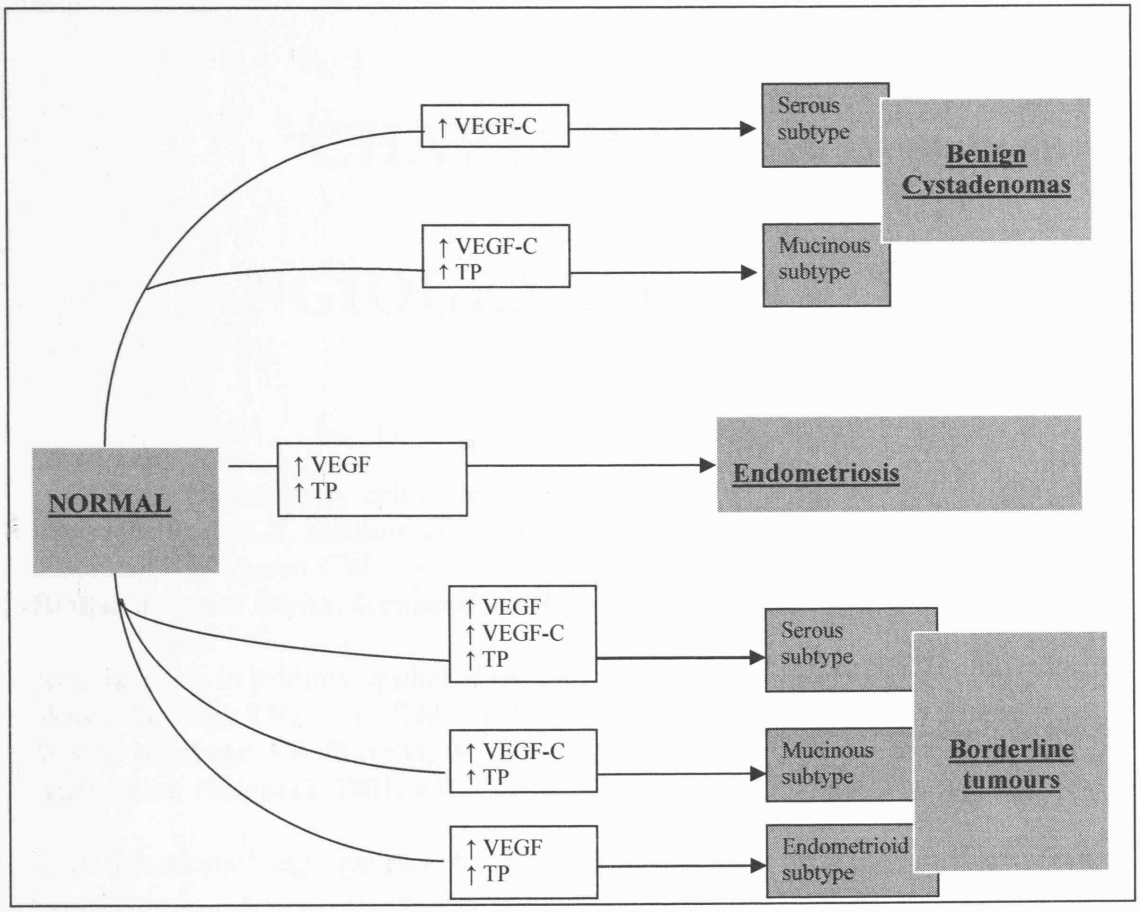



**Figure 3.12 Expression of potential prognostic markers in benign ovarian lesions.**

# CHAPTER FOUR

# ANGIOGENESIS IN EOC

**Abstracts:**

Angiogenesis in primary epithelial ovarian carcinomas.
**Wong Te Fong LF**, Siddiqui GK, Rolfe KJ, Gammell SJ, Crow JC, Reid WMN, MacLean AB, Perrett CW.
**British Journal Obstet Gynaecol 2001, vol 108, p 547-557.**

Angiogenesis in primary epithelial ovarian carcinomas.
**Wong Te Fong LF**, Kini M, Morris R, Rolfe KJ, Gammell SJ, Crow JC, Reid WMN, MacLean AB, Perrett CW.
**Anticancer Research 2001, vol 21, p 1662.**

Quantification of angiogenesis in primary and metastatic epithelial ovarian carcinomas.
**Wong Te Fong LF**, Siddiqui GK, Gammell SJ, Kini M, Crow JC, Bamberger ES, Reid WMN, MacLean AB, Perrett CW.
**British Journal of Cancer June 2002, vol 86, S45.**

**Poster Presentations:**

Angiogenesis in primary epithelial ovarian carcinomas.
**Wong Te Fong LF**, Siddiqui GK, Rolfe KJ, Gammell SJ, Crow JC, Reid WMN, MacLean AB, Perrett CW.
**British Gynaecological Cancer Society, 10-11 November 2000, Portsmouth, UK.**

Angiogenesis in primary epithelial ovarian carcinomas.
**Wong Te Fong LF**, Kini M, Morris R, Rolfe KJ, Gammell SJ, Crow JC, Reid WMN, MacLean AB, Perrett CW.
**IIAR Conference on Apoptosis, 25-28 May 2001, Athens Greece.**

Quantification of angiogenesis in primary and metastatic epithelial ovarian carcinomas.
**Wong Te Fong LF**, Siddiqui GK, Gammell SJ, Kini M, Crow JC, Bamberger ES, Reid WMN, MacLean AB, Perrett CW.
**British Cancer Research, 30 June-3 July 2002, Glasgow UK.**

## 4.1 INTRODUCTION

Ovarian cancer remains the most common gynaecological malignancy in Western countries with 1-2% of all women developing EOC at some time in their lives. It occurs mainly in women over 50 when the ovaries have no physiological role and consequently, abnormal function causes no symptoms. The principal morbidity and mortality is in women with EOC presenting with peritoneal spread, and despite advances in treatment, there has been little change in the mortality rate (http://seer.cancer.gov/faststats/html/inc_ovary.html). Huge efforts worldwide are therefore being made to develop an effective strategy for early detection.

### 4.1.1 CHALLENGE IN SCREENING FOR OVARIAN CANCER

Because a pre-malignant precursor lesion for ovarian cancer has not yet been identified, the challenge of ovarian cancer screening is limited at present to the detection of asymptomatic, early stage disease (Menon and Jacobs, 2002). Ovarian cancer satisfies many of the WHO criteria (Wilson and Jungner, 1968) for population screening. However, it is still unclear whether the currently available screening tests can detect ovarian cancer sufficiently early to allow intervention to alter the natural history of the disease (Jacobs and Menon, 2004). As a result, considerable efforts have been made in the last 20 years to evaluate the tumour marker CA-125 and ultrasound screening for ovarian cancer. Interest in CA-125 as a screening test was initiated by the fact that ~83% of patients with EOC had CA-125 levels ≥35U/ml (Bast et al, 1983; Canney et al, 1994). Elevated levels were found in 50% of women with stage I disease and >90% of women with more advanced stages, though certain tumours (e.g. mucinous and borderline tumours) are likely to be associated with lower CA-125 levels than invasive

serous cancers (Fritsche and Bast, 1998). However one drawback of CA-125 is that it can be elevated in benign diseases and also in non-ovarian cancers (pancreatic, breast, bladder, liver and lung). Uterine leiomyomas, endometriosis, benign ovarian cysts, ectopic pregnancy and physiological conditions such as pregnancy and menstruation can also be associated with raised CA-125 (Sjovall et al, 2002).

### 4.1.2 ANGIOGENIC MARKERS

Angiogenesis, or the growth of new blood vessels, is an essential requirement for tumour growth and neoplasia (Folkman, 1985) and basically depends on the production of angiogenic factors by host and/or tumour cells (Folkman, 1986; 1990). Fast growing tumours contain many new vessels which have less smooth muscle in their walls and therefore provide less resistance to blood flow when compared with vessels associated with benign ovarian tumours. Currently, colour-Doppler ultrasound uses these altered blood flow patterns as markers to differentiate between malignant and benign ovarian masses, however, in a recent trial, 6% of ovarian tumours without blood flow were found malignant (Ueland et al, 2003).

Over the last 15 years, genetic studies in mice, zebrafish and tadpoles have provided insights into the molecular mechanisms that regulate the growth of blood vessels (angiogenesis) or lymph vessels (lymphangiogenesis) in the embryo. In addition, in animal models without angiogenesis, it has been shown that tumours will not expand beyond 2-3 mm in diameter (Folkman, 1986). From these studies, a number of growth factor receptor pathways that promote tumour angiogenesis, have been identified. One of the major pathways involved is the VEGF factor

family of proteins and its receptors. Overexpression of VEGF has been associated with tumour progression and poor prognosis in several tumour systems, including colorectal carcinoma (Takahashi et al, 1996; Lee et al, 2000), gastric carcinoma (Maeda et al, 1996; Takahashi et al, 1996), pancreatic carcinoma ( Fujimoto et al, 1998; Ikeda et al, 1999), breast carcinoma (Manders et al, 2002; Berns et al, 2003), prostate carcinoma (George et al, 2001), lung carcinoma (Fontanini et al, 1997), and malignant melanoma (Gorski et al, 2003). Along with VEGF, the expression of TP, a well-known inducer of angiogenesis, has also been correlated with poor prognosis, if detected at high levels, in human solid tumours such as breast, cervix and uterus (Fox et al, 1996; Fujimoto et al, 1999; Takebayashi et al, 1996).

With regard to ovarian cancer, results relating to angiogenesis as a prognostic marker are still conflicting (Macchiarini et al, 1992; Tanigawa et al, 1996; Weidner et al, 1993). The use of multiple markers has been suggested to increase the sensitivity for early detection of ovarian cancer.

## 4.2 AIMS

In this chapter, the protein expression of VEGF, VEGF-C, VEGFR-1 and TP as well as MVD will be examined to determine whether they can be used as prognostic markers in EOC and their role in malignant progression of this disease.

## 4.3 MATERIALS AND METHODS

### 4.3.1 TISSUE SPECIMENS

Sixty EOC patients, who underwent either hysterectomy or unilateral / bilateral salpingo-oopherectomy, during 1991 to 2001 at the Royal Free Hospital London,

were chosen for this study and 24 normal ovarian specimens obtained from women who underwent oophorectomy for non-ovarian conditions (but related to leiomyomas, uterine bleeding or pelvic pain) were examined as controls (same control group as in previous chapter). Serial sections of 5 μm from formalin-fixed paraffin wax-embedded tissue samples were prepared. H&E section from each tissue was prepared and evaluated by a consultant gynaecological pathologist (JCC) to confirm the original diagnosis. Histological parameters of these tumours were determined using the WHO criteria (Serov et al, 1973) and are summarised in Table 4.1. Because clear cell tumours are thought to be related to endometrioid tumours and because the numbers in these groups were low, these two subtypes were analysed together for statistical comparisons with other subtypes.

| **Diagnosis of EOC** | **Number (n)** |
|---|---|
| **Subtypes** | |
| **Serous** | 31 |
| **Mucinous** | 11 |
| **Endometrioid and clear cell (endo-cc)** | 12 (8 and 4 respectively) |
| **Mixed** | 3 |
| **Unclassified** | 3 |
| **Stages** | |
| **I** | 9 |
| **II** | 4 |
| **III** | 42 |
| **IV** | 3 |
| **Unclassified** | 2 |
| **Grades** | |
| **1** | 6 |
| **2** | 17 |
| **3** | 29 |
| **Unclassified** | 8 |

**Table 4.1 EOC patients' histological characteristics.**

### 4.3.2 IMMUNOHISTOCHEMICAL ANALYSIS

IHC was performed to detect the protein expression of VEGF, VEGF-C, VEGFR-1, TP and vWF (for MVD analysis) and repeated three times to ensure consistency as described in chapter 2, Section 2.2.1.3 and 2.2.1.4 respectively. Scoring of each marker was then performed (see Table 2.2, Section 2.2.1.5).

### 4.3.3 STATISTICAL ANALYSIS

The angiogenic proteins expression including VEGF, VEGF-C, VEGFR-1 and TP in relation to EOC various histological parameters were assessed using the chi-square test and Fisher's exact test. Comparison between the mean MVD values between the EOC histological parameters was analysed by the Independent sample t-test. Correlation between angiogenic proteins and MVD was examined by Spearman rank correlation test. A probability with a p value $<0.05$ was considered significant.

## 4.4 RESULTS

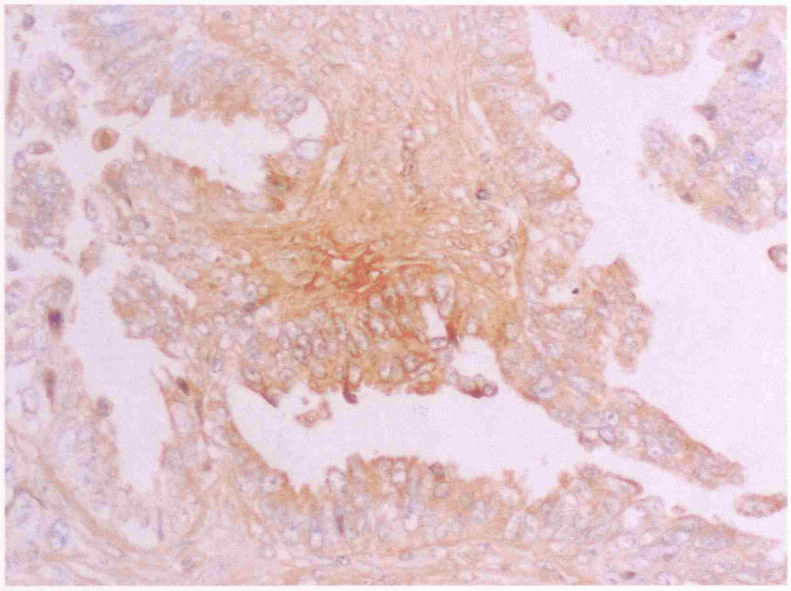
Table 4.2 gives the mean age of these EOC patients according to their histological parameters and there was no significant difference between them. However, when the mean age of all these EOC patients, at surgery (58.8 ± 13.9, range 25-86) years was compared with controls (49.8 ± 11.3 years, 25-81), this difference was significant ($p=0.006$). This is in accord with the fact that women presenting with EOC are generally diagnosed late, i.e. at a later age in their life.

| EOC diagnosis | n | Age in years mean ± SD (range) | p value |
|---|---|---|---|
| **Subtypes** | | | |
| **Serous** | 31 | 57.2 ± 11.8 (36-79) | 0.77 (serous vs mucinous) |
| **Mucinous** | 11 | 55.9 ± 17.1 (25-86) | 0.56 (mucinous vs endo-cc) |
| **Endo-cc** | 12 | 59.9 ± 15.7 (39-84) | 0.54 (endo-cc vs serous) |
| **Mixed** | 3 | 67.3 ± 16.7 (48-77) | ~0.55 (mixed vs each other subtype) |
| **Unclassified** | 3 | 70.5 ± 11.8 (54-82) | - |
| **Stages** | | | |
| **I** | 9 | 52.2 ± 19.0 (25-84) | 0.24 (I vs II) |
| **II** | 4 | 66.0 ± 17.6 (42-83) | 0.26 (II vs III) |
| **III** | 42 | 58.3 ± 12.4 (36-86) | 0.24 (I vs III) |
| **IV** | 2 | 65.0 ± 4.6 (61-70) | ~0.36 (IV vs each other stage) |
| **Unclassified** | 2 | 71.3 ± 12.2 (54-82) | - |
| **Grades** | | | |
| **1** | 6 | 51.7 ± 7.1 (42-60) | 0.19 (1 vs 2) |
| **2** | 17 | 60.4 ± 14.9 (39-84) | 0.64 (2 vs 3) |
| **3** | 29 | 58.5 ± 13.1 (36-83) | 0.23 (1 vs 3) |
| **Unclassified** | 8 | 61.6 ± 19.8 (25-86) | - |

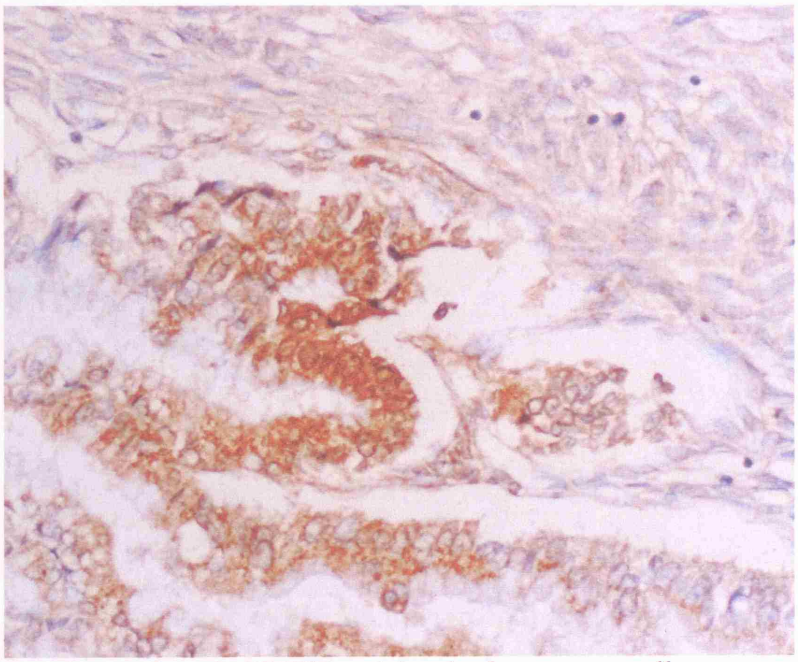
**Table 4.2 Mean age in EOC patients according to histological features.**

### 4.4.1 VEGF EXPRESSION AND EOC

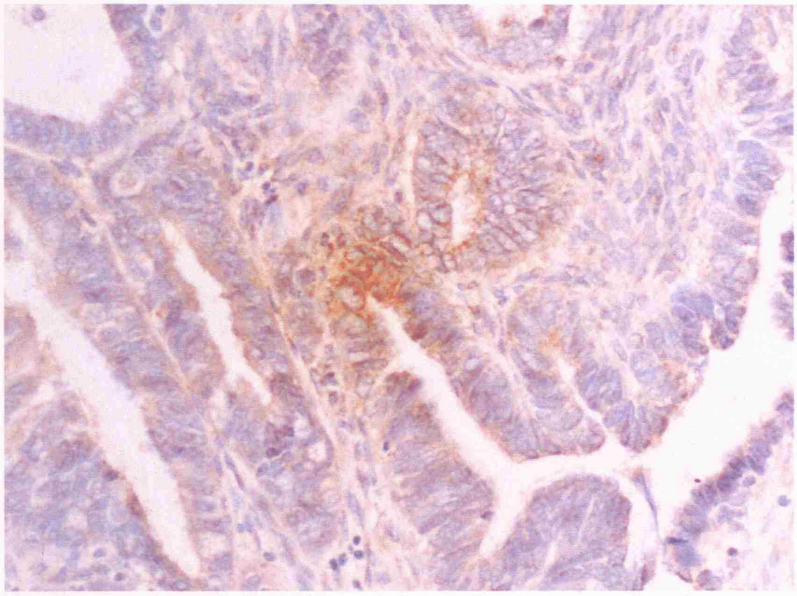
Positive focal areas of VEGF expression (Figure 4.1) were found in the cytoplasm of both epithelial tumour cells and stromal cells and occasionally on the membranes of the tumour cells, in the different subtypes of EOC.



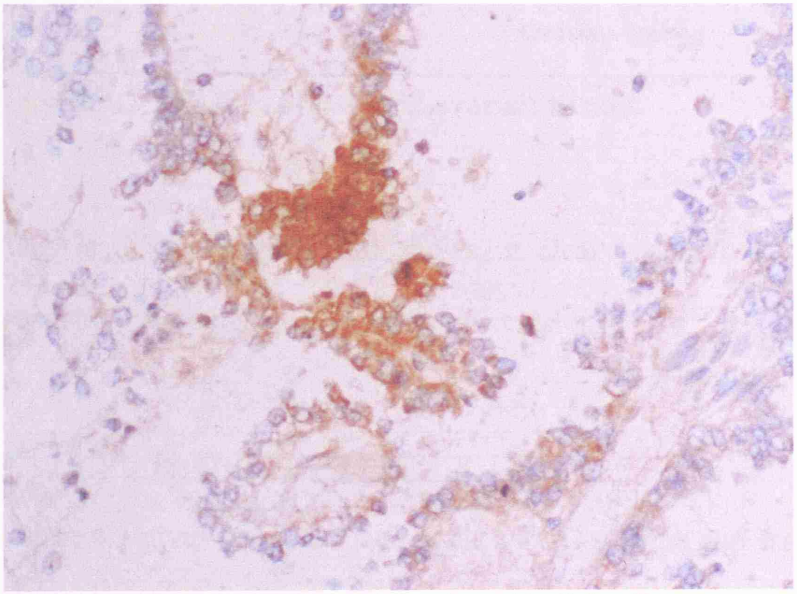
Focal cytoplasmic VEGF staining in the stromal cells of the tumour.
**Figure 4.1a VEGF in serous EOC (X200).**



Focal cytoplasmic VEGF staining in the tumour cells.
**Figure 4.1b VEGF in mucinous EOC (X200).**



Focal cytoplasmic VEGF staining in both stromal and tumour cells.
**Figure 4.1c VEGF in endometrioid EOC (X200).**



Focal cytoplasmic VEGF staining in epithelial cells.
**Figure 4.1d VEGF in clear-cell EOC (X200).**

When all EOC were grouped initially, a significantly higher number of EOC were positive for VEGF compared to controls and benign cystadenomas (p=0.0001 and 0.003 respectively) but there was no significant difference between EOC and borderline tumours or endometriotic lesions (p=0.09 and 0.08 respectively, although more endometriotic lesions expressed VEGF compared to EOC.

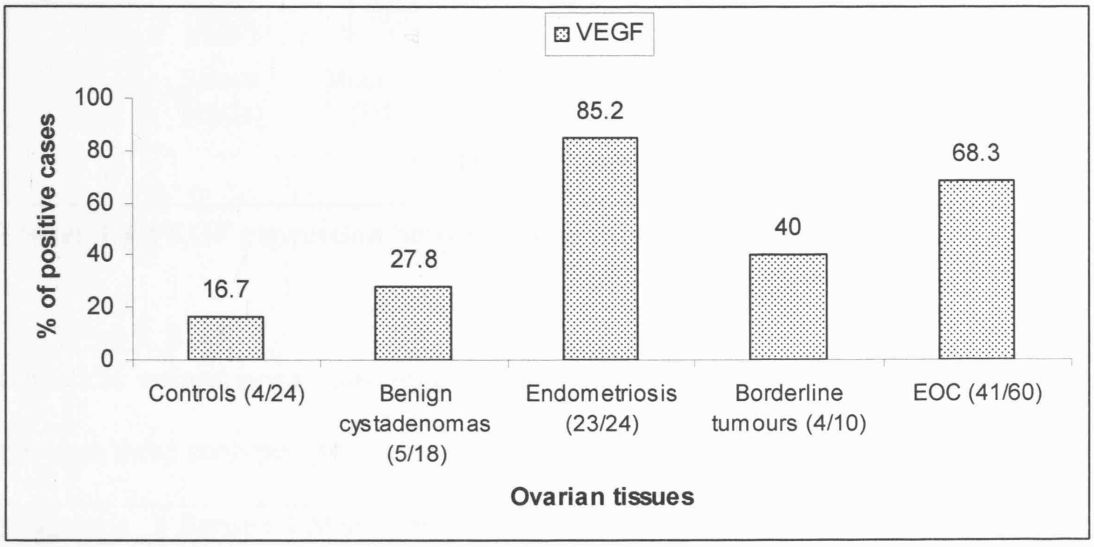



**Figure 4.2 VEGF expression in ovarian tissues.**

This is a key diagram that shows in clear visual form an interesting result separating endometriosis from the other lesions.

4.4.1.1 VEGF AND EOC TUMOUR TYPE

Figure 4.3 gives the percentage of each EOC subtype expressing VEGF. A higher proportion of endometrioid and clear-cell EOC expressed VEGF compared to other subtypes.

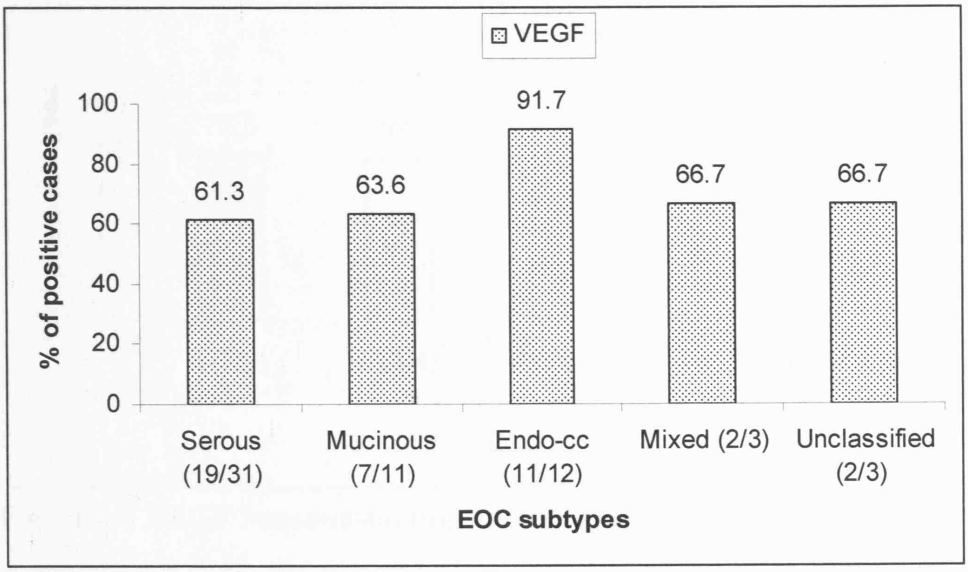



**Figure 4.3 VEGF expression between EOC subtypes.**

Statistical comparisons showed no significant difference in VEGF expression between these subtypes (see Table 4.3).

| Diagnosis | Serous | Mucinous | Endo-cc |
|---|---|---|---|
| **Mucinous** | 0.89 | | |
| **Endo-cc** | 0.14 | 0.24 | |
| **All others** | 0.97 | 0.99 | 0.55 |

**Table 4.3 P values comparing VEGF expression for each tumour type.**

4.4.1.2 VEGF AND EOC TUMOUR STAGE

VEGF immunostaining was then compared between EOC tumour stage and the results are summarised in Figure 4.4.

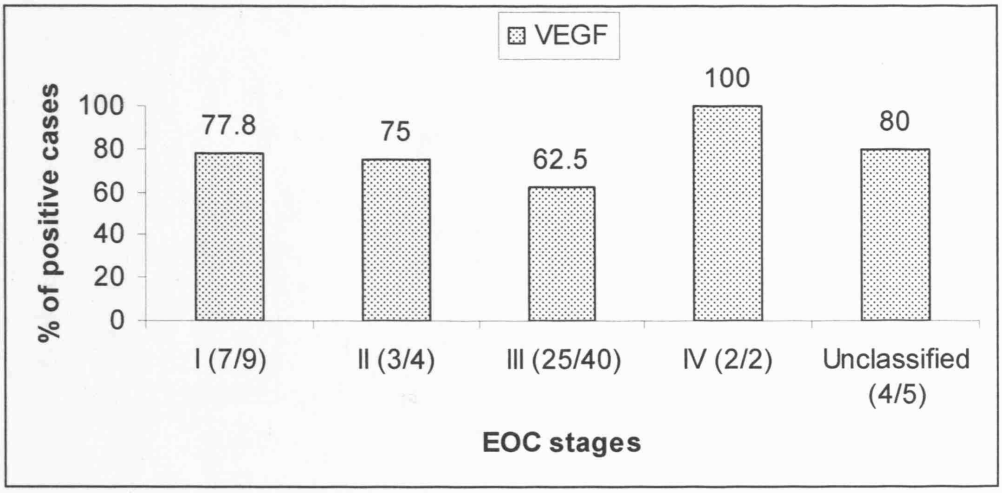



**Figure 4.4 VEGF immunostaining according to EOC tumour stages.**

The results of the statistical analysis for the comparison between VEGF protein expression in EOC grouped according to tumour stage are shown in Table 4.4, and no differences were found in VEGF immunostaining between the various tumour stages. For a better statistical analysis, stages I and II were also combined together and compared with stages III with IV. 76.9% (10/13) of stages I-II were VEGF positive and 64.3% (27/42) of stages III-IV, but this difference was also not significant (p=0.31).

| **Stage** | **I** | **II** | **III** |
|---|---|---|---|
| **II** | 0.71 | | |
| **III** | 0.32 | 0.54 | |
| **IV** | 0.66 | 0.67 | 0.41 |

**Table 4.4 P values comparing VEGF staining between each tumour stage.**

4.4.1.3 VEGF AND EOC TUMOUR GRADE

Figure 4.5 shows more grade 2 tumours expressing VEGF compared to the other tumour grades.

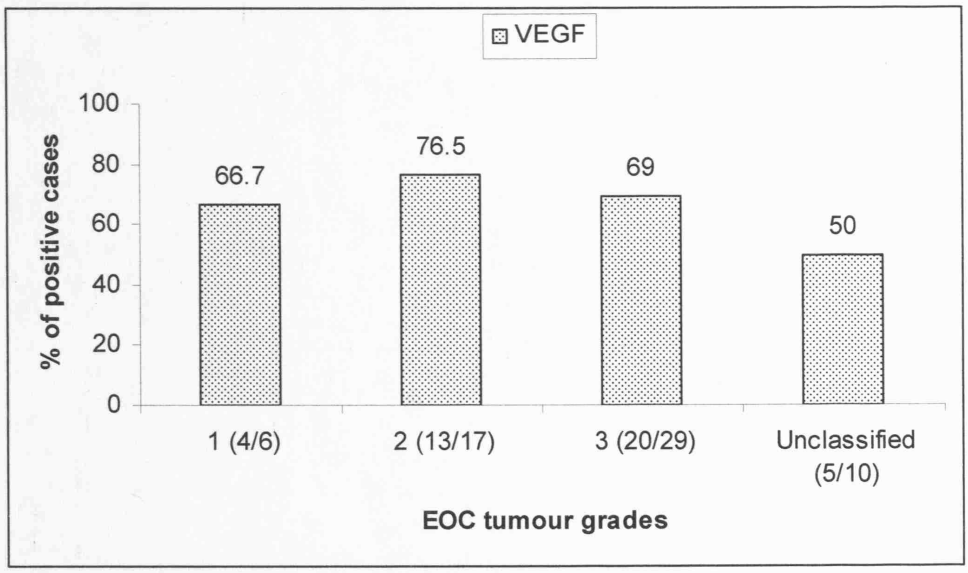



**Figure 4.5 VEGF immunostaining according to EOC tumour grades.**

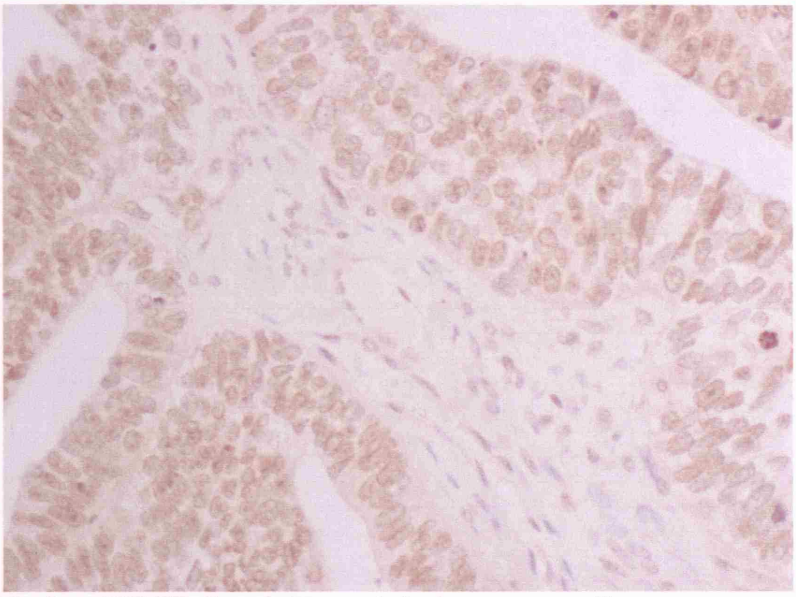
The p values obtained by statistical analysis did not show any significant difference between VEGF expression and tumour grade, as summarised in Table 4.5.

| Grade | 1 | 2 |
|---|---|---|
| 2 | 0.51 | |
| 3 | 0.63 | 0.42 |

**Table 4.5 P values comparing VEGF expression between each tumour grade.**

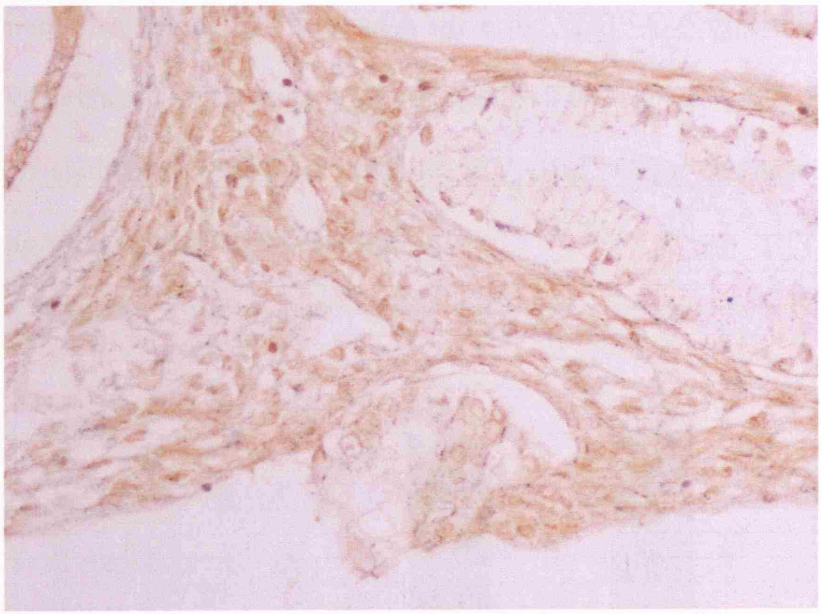
### 4.4.2 VEGF-C EXPRESSION AND EOC

VEGF-C expression is expressed in the cytoplasm and nuclei of both epithelial and stromal cells in different EOC subtypes (Figure 4.6)



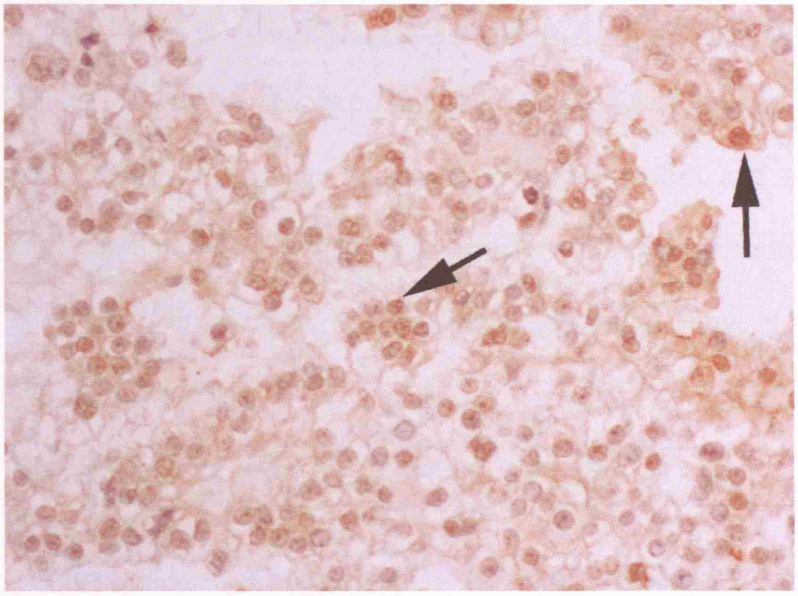
Nuclear VEGF-C staining mainly in tumour cells

**Figure 4.6a VEGF-C in serous EOC (X200).**



Mainly cytoplasmic VEGF-C staining in the stroma of the tumour.

**Figure 4.6b VEGF-C in mucinous EOC (X200).**



Arrows show nuclear VEGF-C staining of tumour cells.
**Figure 4.6c VEGF-C in clear cell EOC (X200).**

Figure 4.7 shows that out of the 51 EOC specimens available for VEGF-C immunostaining, 84.3% were positive for VEGF-C compared to controls and this difference was statistically significant (p=0.0001, Figure 4.6). However, there was no significant difference between EOC and benign cystadenomas or borderline tumours (p=0.56 and 0.54 respectively).

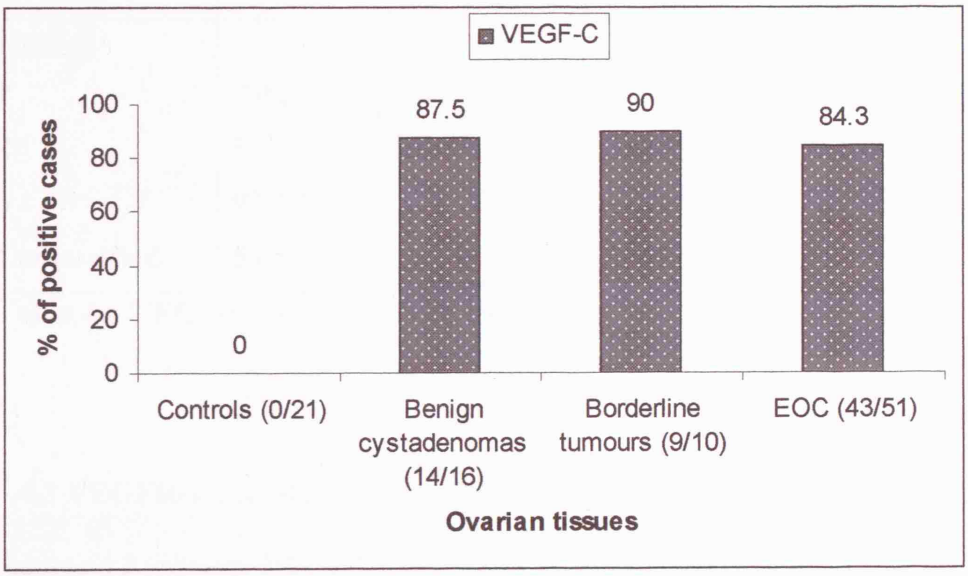



**Figure 4.7 VEGF-C expression in EOC versus controls.**

4.4.2.1 VEGF-C AND EOC HISTOLOGICAL PARAMETERS

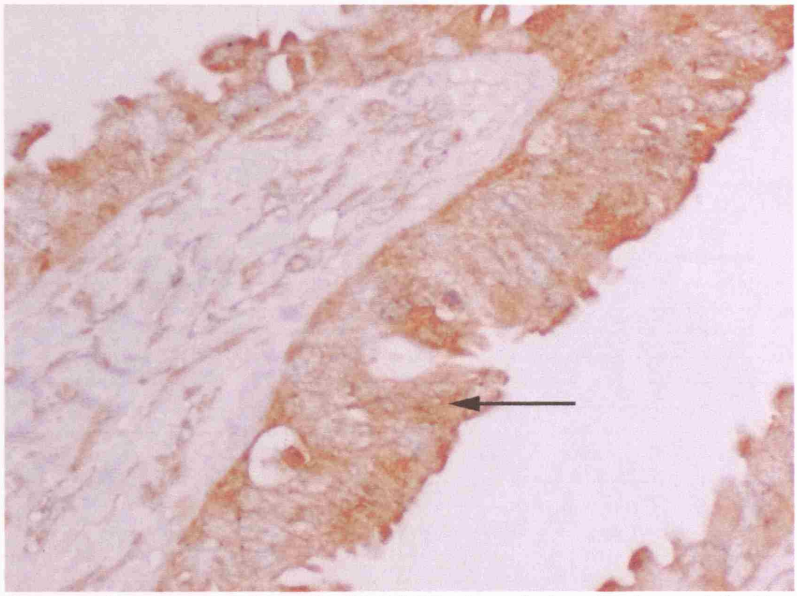
Table 4.6 shows the expression of VEGF-C according to EOC histological parameters. Among the subtypes, all endometrioid and clear cell carcinomas expressed VEGF-C compared to serous and mucinous subtypes, but this was not significant. In addition, no correlation was found between VEGF-C and tumour stages or grades.

| EOC diagnosis | VEGF-C positive | p value |
|---|---|---|
| **Subtypes** | | |
| **Serous** | 81.5% (22/27) | 0.63 (serous vs mucinous) |
| **Mucinous** | 80% (8/10) | 0.33 (mucinous vs endo-cc) |
| **Endo-cc** | 100% (10/10) | 0.34 (endo-cc vs serous) |
| **Mixed** | 66.7% (2/3) | ~0.73 (mixed vs each other subtype) |
| **Unclassified** | 100% (1/1) | - |
| **Stages** | | |
| **I** | 100% (9/9) | 0.22 (I vs III) |
| **II** | 100% (3/3) | 0.58 (II vs III), 0.50 (II vs IV) |
| **III** | 82.4% (28/34) | 0.48 (III vs IV) |
| **IV** | 66.7% (2/3) | 0.25 (I vs IV) |
| **Unclassified** | 50% (1/2) | - |
| **Grades** | | |
| **1** | 75% (3/4) | 0.60 (1 vs 2) |
| **2** | 82.4% (14/17) | 0.32 (2 vs 3) |
| **3** | 92% (23/25) | 0.37 (1 vs 3) |
| **Unclassified** | 50% (2/4) | |

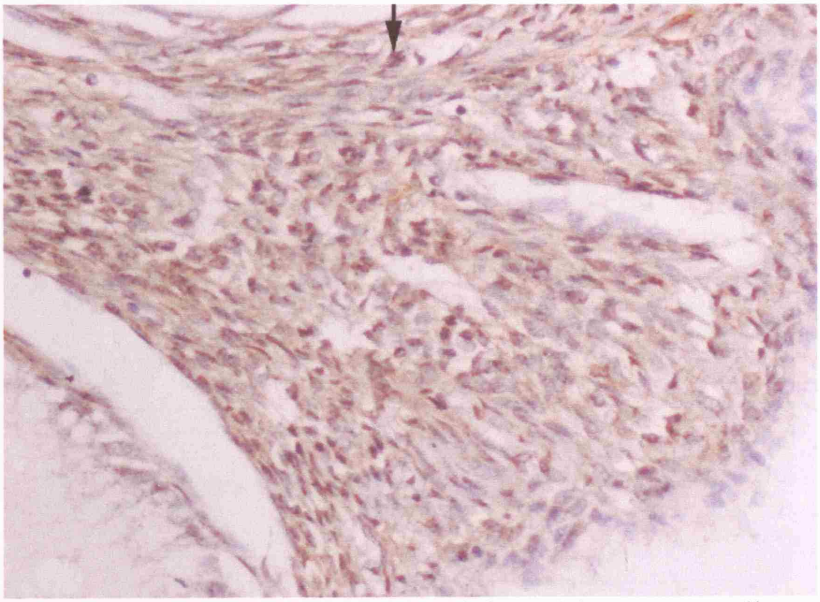
**Table 4.6 VEGF-C expression according to EOC histological features.**

### 4.4.3 VEGFR-1 EXPRESSION AND EOC

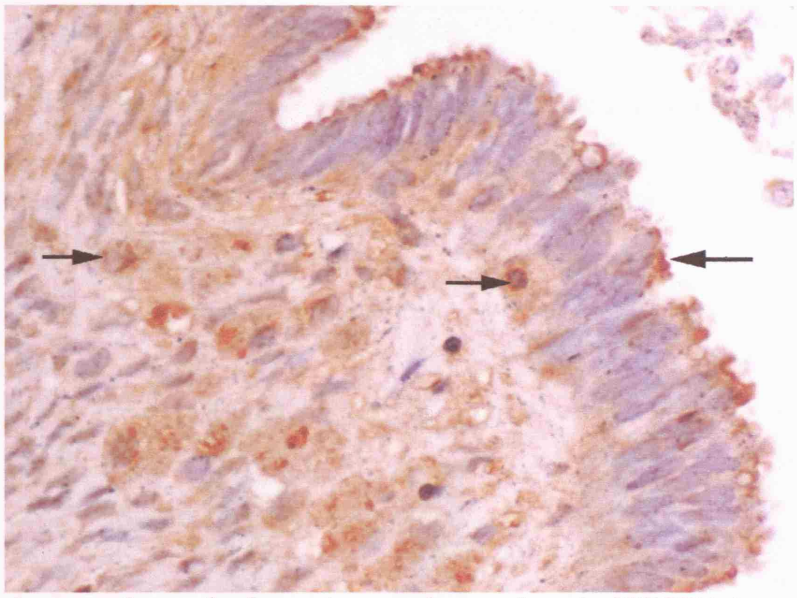
Figure 4.8 shows VEGFR-1 expression in different EOC subtypes, either cytoplasmic or nuclear, mainly in the tumour cells.



Arrow shows cytoplasmic VEGFR-1 staining in the epithelium of the tumour.
**Figure 4.8a VEGFR-1 in serous EOC (X200).**

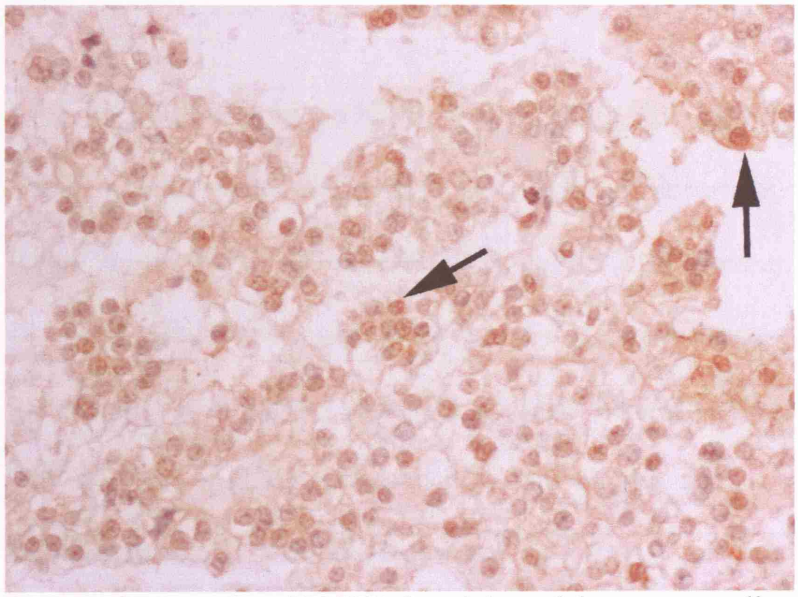


Arrow shows nuclear VEGFR-1 staining of the tumour cells.
**Figure 4.8b VEGFR-1 in mucinous EOC (X200).**



Arrows shows both nuclear and cytoplasmic VEGFR-1 staining in stroma and epithelium as well as on surface of epithelial cells.

**Figure 4.8c VEGFR-1 in endometrioid EOC (X200).**



Arrows show nuclear VEGFR-1 staining of the tumour cells.

**Figure 4.8d VEGFR-1 in clear-cell EOC (X200).**

Figure 4.9 shows that out of the 40 EOC specimens available for VEGFR-1 immunostaining, 92.5% were positive. When a comparison was made between EOC and controls or benign ovarian neoplasm, no significant difference was found (p~0.65).

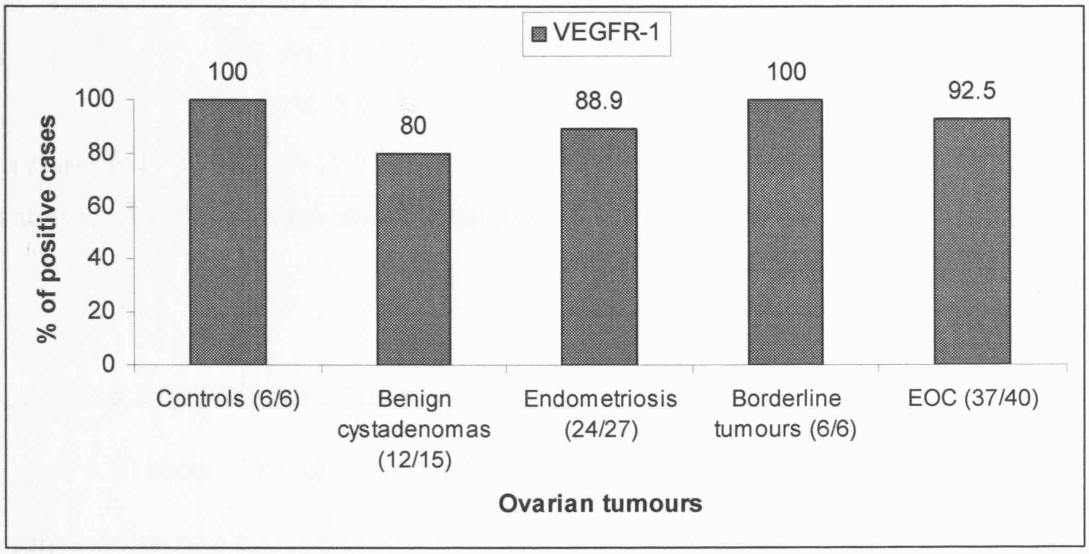



**Figure 4.9 VEGFR-1 expression in EOC versus controls.**

4.4.3.1 VEGFR-1 AND EOC HISTOPATHOLOGICAL PARAMETERS

Table 4.7 shows the expression of VEGFR-1 according to EOC histological features. Like VEGF-C, VEGFR-1 expression was present in all endometrioid and clear cell carcinomas compared to serous and mucinous subtypes, but this was not significant. In addition, statistical analysis between VEGFR-1 expression and tumour stages or grades did not achieve any significance.

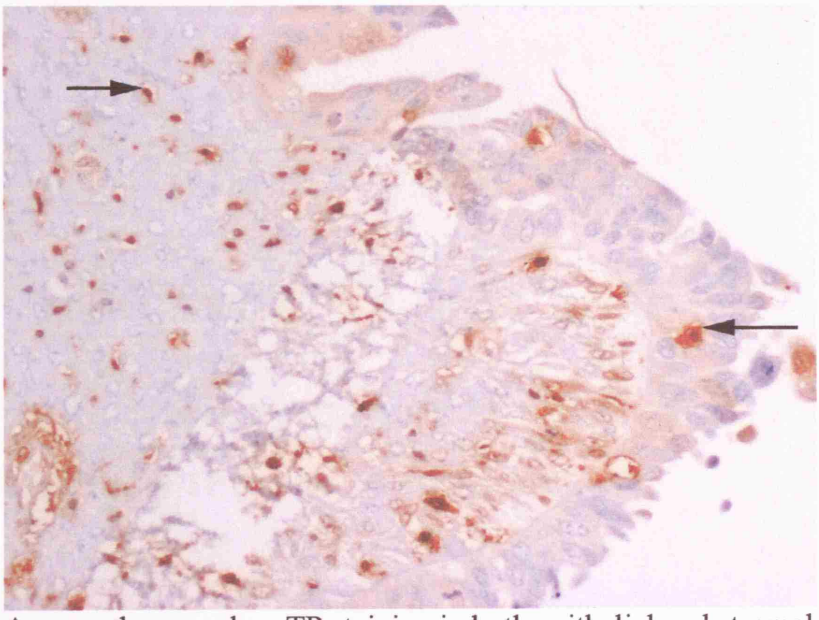
| EOC diagnosis | VEGFR-1 positive | p value |
|---|---|---|
| **Subtypes** | | |
| **Serous** | 88.9% (16/18) | 0.68 (serous vs mucinous) |
| **Mucinous** | 87.5% (7/8) | 0.44 (mucinous vs endo-cc) |
| **Endo-cc** | 100% (10/10) | 0.52 (endo-cc vs serous) |
| **Mixed** | 100% (3/3) | ~0.89 (mixed vs serous or mucinous) |
| **unclassified** | 100% (1/1) | - |

| | | |
|---|---|---|
| **Stages** | | |
| **I** | 8 (100%) | 0.45 (I vs III) |
| **II** | 2 (100%) | 0.80 (II vs III) |
| **III** | 24 (88.9%) | - |
| **Grades** | | |
| **1** | 75% (3/4) | 0.48 (1 vs 2) |
| **2** | 90.9% (10/12) | 0.61 (2 vs 3) |
| **3** | 94.7% (18/19) | 0.32 (1 vs 3) |
| **unclassified** | 100% (6/6) | - |

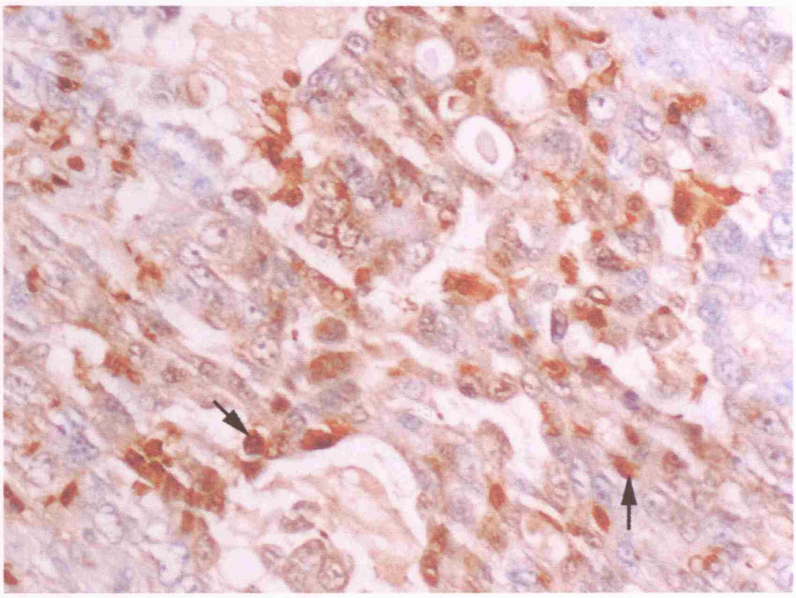
**Table 4.7 VEGFR-1 expression according to EOC histological features.**

### 4.4.4 TP EXPRESSION AND EOC

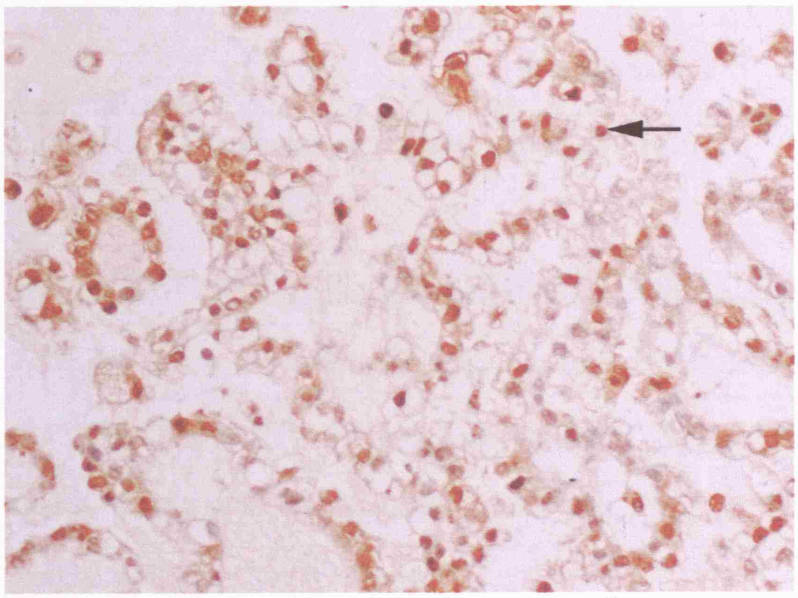
Figure 4.10 shows TP expression in different EOC subtypes. Cells positive for TP expression show a common pattern of cytoplasmic and nuclear staining located in the epithelial and stromal cells of tumours which is often enhanced at the tumour edge (Figure 4.10 a).



Arrows show nuclear TP staining in both epithelial and stromal cells
**Figure 4.10a TP in serous EOC (X200).**



Arrows show nuclear TP staining in tumour cells.
**Figure 4.10b TP in mucinous EOC (X200).**



Arrow shows nuclear TP staining in tumour cells.
**Figure 4.10c TP in clear cell EOC (X200).**

Figure 4.11 shows a significantly higher number of EOC cases positive for TP compared to controls and benign cystadenomas (p=0.0001 and 0.02 respectively) but a significantly lower proportion of EOC positive for TP compared to endometriotic lesions (p=0.01) and no significant difference in TP expression between EOC and borderline tumours (p=0.61). Again, the endometriotic cases stand out as different.

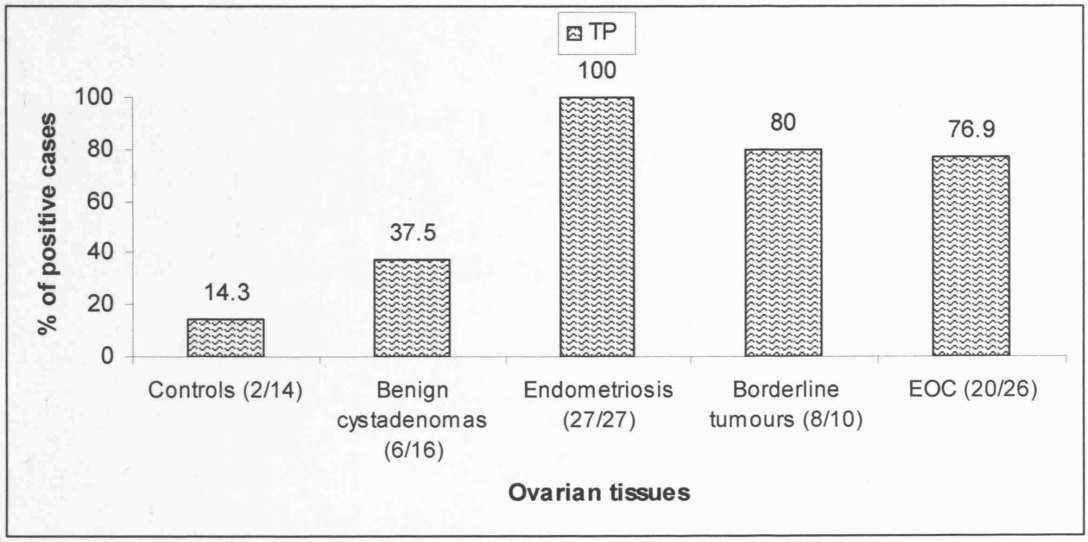



**Figure 4.11 TP expression in EOC versus controls.**

4.4.4.1 TP EXPRESSION AND EOC HISTOLOGICAL PARAMETERS

Table 4.8 shows the histopathological characteristics of the EOC patients for TP expression. No correlation was found between TP and histological subtypes or stages, however, a significant difference was found in TP expression between grade 2 and grade 3 EOC tumours.

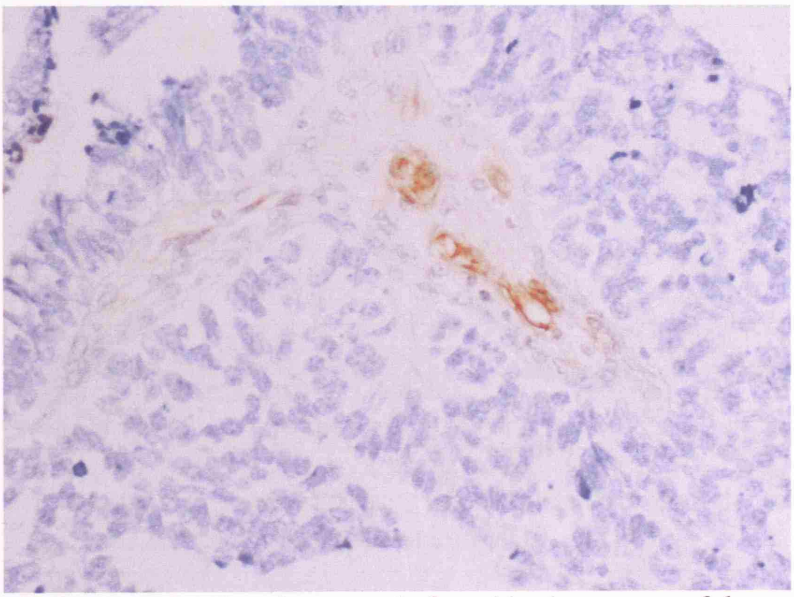
| EOC diagnosis | TP positive | p value |
|---|---|---|
| **Subtypes** | | |
| **Serous** | 78.6% (11/14) | 0.54 (serous vs mucinous) |
| **Mucinous** | 100% (3/3) | 0.42 (mucinous vs endo-cc) |
| **Endo-cc** | 66.7% (4/6) | 0.48 (endo-cc vs serous) |
| **Mixed** | 50% (1/2) | ~0.55 (mixed vs each other subtype) |

| | | |
|---|---|---|
| **unclassified** | 100% (1/1) | - |
| **Stages** | | |
| **I** | 33.3% (1/3) | 0.70 (I vs II) |
| **II** | 50% (1/2) | 0.28 (II vs III) |
| **III** | 88.9% (16/18) | 0.08 (I vs III) |
| **Grades** | | |
| **2** | 40% (2/3) | **0.01** (2 vs 3) |
| **3** | 100% (13/13) | |

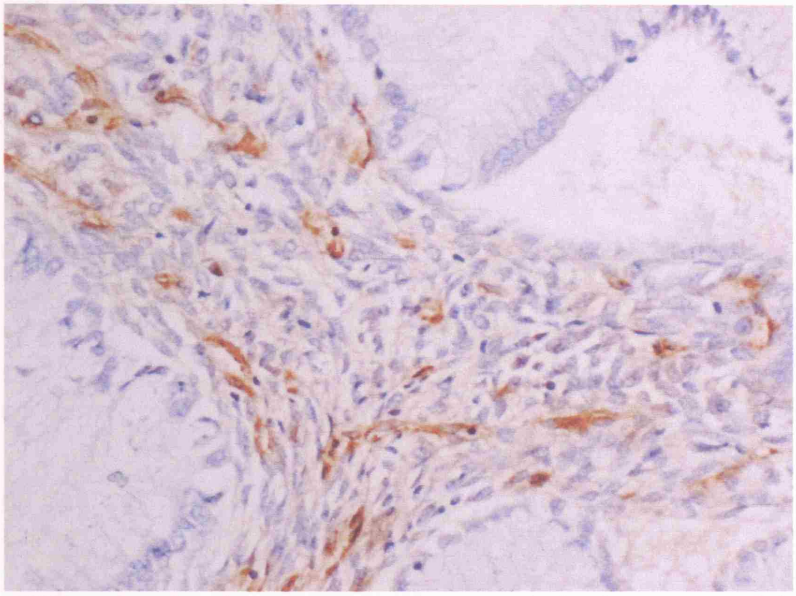
**Table 4.8 TP expression according to EOC histopathological features.**

### 4.4.5 MVD AND EOC

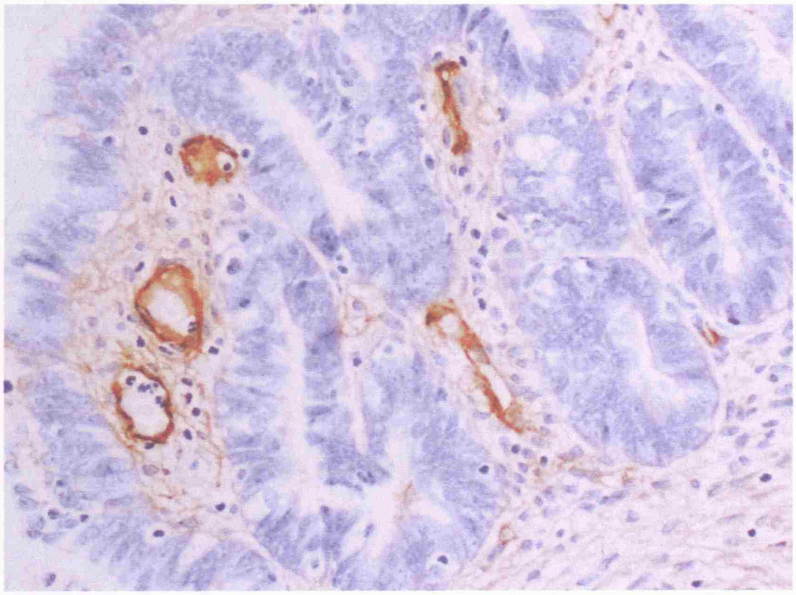
Figure 4.12 represents immunohistochemical staining of endothelial cells with anti-vWF antibody on EOC of different subtypes. The anti-vWF antibody stained the vascular endothelial cells brown (immunoperoxidase stain) with variable intensities.



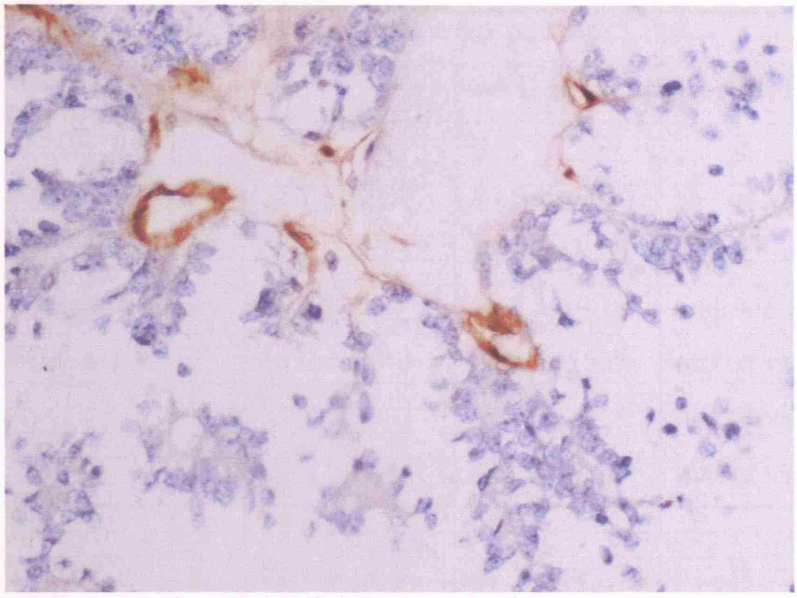
vWF expression in microvessels found in the stroma of the tumour.
**Figure 4.12a MVD in serous EOC (X200).**



vWF expression in numerous microvessels in the stroma of the tumour.
**Figure 4.12b MVD in mucinous EOC (X200).**



vWF expression in microvessels found in the stroma of the tumour.
**Figure 4.12c MVD in endometrioid EOC (X200).**



vWF expression highlighting several microvessels in the stroma of the tumour.
**Figure 4.12d MVD in clear-cell EOC (X200).**

From Table 4.9, the mean HVD and AVD values per $mm^2$ in all EOC samples were 57.8 ± 23.3 and 47.0 ± 17.8 respectively. When compared with HVD and AVD in controls, the difference between EOC and controls was significant (p=0.0001 and 0.0001 respectively).

| **Diagnosis** | **n** | **Mean MVD ± SD values per $mm^2$** | |
|---|---|---|---|
| | | **HVD** | **AVD** |
| **Controls** | 23 | 80.8 ± 19.9 | 67.1 ± 16.1 |
| **EOC** | 47 | 57.8 ± 23.3 | 47.0 ± 17.8 |

**Table 4.9 MVD values in EOC versus controls.**

4.4.5.1 MVD AND EOC HISTOLOGICAL PARAMETERS

Comparisons of the mean HVD and AVD values, classified according to EOC histological features are given in Table 4.10. No significant difference was found in MVD between tumour subtypes or grades. Stage III tumours show a significant decrease in MVD compared to stage I tumours, possibly because more of the tumour is occupied by malignant epithelium rather than stroma with vessels.

| EOC diagnosis | n | Mean MVD ± SD per $mm^2$ | | p value | | |
|---|---|---|---|---|---|---|
| | | HVD | AVD | Correlations | HVD | AVD |
| **Histotype** | | | | | | |
| **Serous** | 26 | 55.2 ± 21.7 | 44.6 ± 17.0 | serous vs mucinous | 0.35 | 0.33 |
| **Mucinous** | 7 | 64.6 ± 30.3 | 52.1 ± 20.2 | mucinous vs endo-cc | 0.74 | 0.79 |
| **Endo-cc** | 9 | 60.0 ± 23.5 | 49.5 ± 19.1 | endo-cc vs serous | 0.58 | 0.48 |
| **Mixed** | 3 | 45.0 ± 21.0 | 35.8 ± 15.6 | mixed vs serous | 0.45 | 0.40 |
| | | | | mixed vs mucinous | 0.34 | 0.25 |
| | | | | mixed vs endo-cc | 0.35 | 0.30 |
| **Stages** | | | | | | |
| **I** | 8 | 74.9 ± 15.6 | 61.1 ± 11.6 | I vs II | 0.96 | 0.60 |
| **II** | 3 | 74.3 ± 38.3 | 55.6 ± 22.6 | II vs III | 0.19 | 0.23 |
| **III** | 30 | 51.8 ± 38.2 | 42.3 ± 17.3 | I vs III | **0.008** | **0.006** |
| **Grades** | | | | | | |
| **1** | 3 | 72.0 ± 39.4 | 54.5 ± 21.5 | 1 vs 2 | 0.81 | 0.92 |
| **2** | 14 | 68.0 ± 22.1 | 55.6 ± 17.1 | 2 vs 3 | 0.03 | 0.02 |
| **3** | 21 | 51.3 ± 22.8 | 40.9 ± 18.0 | 1 vs 3 | 0.19 | 0.25 |

**Table 4.10 MVD according to EOC histological parameters.**

### 4.4.6 DOUBLE MARKER EXPRESSION

Table 4.11 shows the correlation of two markers between controls and EOC. Compared to normal ovaries (controls), EOC shows some significant differences (i.e. statistically significant) when analysing two markers at the same time.

| Double marker staining | Controls | EOC | p value |
|---|---|---|---|
| **VEGF (+) and VEGF-C (+)** | 0 | 26/48 (54.2%) | **0.0001** |
| **VEGF (+) and VEGFR-1 (+)** | 0 | 25/40 (62.5%) | **0.011** |
| **VEGF (+) and TP (+)** | 0 | 8/26 (30.8%) | **0.0001** |

**Table 4.11 Correlation of double markers between EOC and controls.**

### 4.4.7 PROTEIN EXPRESSION AND MVD

Table 4.12 shows that MVD values in VEGF-positive tumours are significantly higher than that in VEGF-negative tumours of EOC; however, MVD values are lower in VEGF-C, VEGFR-1 and TP-positive tumours compared to that of their corresponding negative counterparts, and no statistically significant difference was found.

| Protein expression | n | Mean MVD ± SD per $mm^2$ | | p value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| VEGF (-) | 18 | 46.8 ± 21.3 | 38.3 ± 17.4 | | |
| VEGF (+) | 27 | 65.1 ± 22.3 | 53.1 ± 16.02 | **0.008** | **0.005** |
| VEGF-C (-) | 5 | 68.0 ± 20.0 | 54.2 ± 11.8 | | |
| VEGF-C (+) | 33 | 56.2 ± 24.6 | 45.3 ± 18.9 | 0.313 | 0.317 |
| VEGFR-1 (-) | 4 | 65.1 ± 30.8 | 54.7 ± 21.0 | | |
| VEGFR-1 (+) | 33 | 56.9 ± 23.5 | 46.1 ± 17.8 | 0.53 | 0.38 |
| TP (-) | 6 | 66.2 ± 15.8 | 57.9 ± 15.3 | | |
| TP (+) | 22 | 47.3 ± 20.9 | 38.6 ± 16.4 | 0.05 | **0.02** |

**Table 4.12 Correlation between MVD values in relation to the positivity of angiogenic proteins, in EOC.**

## 4.5 DISCUSSION

### 4.5.1 VEGF IN EOC

Table 4.13 shows some of the recent studies assessing VEGF in ovarian carcinomas; with the majority of them showing a high proportion of EOC cases expressing VEGF.

| Authors | Results |
|---|---|
| Abu-Jawdeh et al, 1996 | The malignant epithelium stained for VEGF protein in all EOC cases. |
| Boocock et al, 1995 | VEGF mRNA detected in 3 of 4 ovarian carcinoma cells |

| | |
|---|---|
| | lines and all 4 ovarian tumours expressed VEGF proteins. |
| Brustmann and Naude, 2002 | VEGF was positive in 41 of 45 EOC cases and FIGO stage III cases displayed stronger VEGF staining than stage I and II taken together. |
| Brustmann, 2004 | 51% (21/41) EOC had focal or diffuse strong VEGF immunostaining. Correlations between VEGF and disease stage and grade. |
| Chen et al, 2004 | VEGF in ovarian carcinomas higher than that in benign and normal ovarian tissues. |
| Fujimoto et al, 1998 | Levels of VEGF and VEGF165 mRNA in ovarian carcinomas were significantly higher than in normal ovaries. |
| Inan et al, 2006 | VEGF expression higher in malignant than in borderline tumours. |
| Karavasilis et al, 2006 | VEGF was strong in 91% of 33 patients with FIGO III ovarian cancer. No correlation between VEGF and tumour grade, type and survival. |
| Li et al, 2005 | 92.6% of 27 malignant ovarian tumours expressed VEGF. |
| Nakanishi et al, 1997 | 78.3% of 60 EOC were positive for VEGF. No correlation between VEGF and FIGO stage. But VEGF was significantly stronger in mucinous EOC than in endometrioid and clear cell carcinomas. |
| Nishida et al, 2004 | VEGF lower in mucinous carcinomas than other subtypes but this was not significant. |
| Ogawa et al, 2002 | Strongly positive VEGF expression was observed in 49 of 105 EOC. |
| Orre and Rogers, 1999 | VEGF immunostaining did not differ between ovarian tumour types. |
| Shen et al, 2000 | 48% (31/64) had high VEGF expressions. Correlations between VEGF and diseases stage, grade and patient's outcome. |
| Sonmezer et al, 2004 | No correlation between VEGF and clinicopathological factors or survival. |

| | |
|---|---|
| Sowter et al, 1997 | VEGF mRNA present in all ovarian tissues examined. |
| Sundar et al, 2006 | Strong staining of VEGF noted in 29% (epithelial), 17% (stromal), 43% (macrophage) and 19% (vascular) of 108 EOC tumours. |
| Wang et al, 2002 | VEGF mRNA detected in 80% of ovarian carcinomas. |
| Yamamoto et al, 1997 | VEGF was positive in 97% of 70 ovarian carcinomas and frequently observed in clear cell EOC tumours and advanced stage of the disease. |

**Table 4.13 Studies of VEGF in ovarian carcinomas.**

In this study, focal and sometimes diffuse strong immunostaining of VEGF were found in 68.3% of the 60 EOC cases analysed compared to 16.7% of 24 normal ovarian tissues. This implies that angiogenesis is stimulated during EOC progression.

No correlation was found between VEGF expression and tumour type / stage / grade in the EOC samples investigated here. This finding agrees with several authors (Nakanishi et al; 1997; Sonmezer et al, 2004; Karavasilis et al, 2006) in which they also did not find any correlation with such clinicopathological parameters. In contrast, others reported a significant correlation of VEGF with histological grade, disease stage and patient outcome (Shen et al, 2000; Brustmann, 2004). Nakanishi et al (1997) found that mucinous adenocarcinomas had a significantly stronger intensity for VEGF than endometrioid and clear cell carcinomas, however in this study, a high proportion of endometrioid and clear cell carcinomas (91.7%) stained positively for VEGF than the other subtypes. In addition, a high proportion of endometriosis (85.2%) also expressed VEGF and this difference was significant between EOC and endometriosis. These findings

clearly indicate the importance of angiogenesis in endometriosis, endometrioid and clear cell carcinomas.

### 4.5.2 VEGF-C IN EOC

In spite of the large amount of data regarding VEGF in ovarian neoplasms, there are few reports on VEGF-C expression as a diagnostic marker in human neoplasms (see Table 4.14).

| Authors | Results |
|---|---|
| Nishida et al, 2004 | 72.5% of 80 EOC expressed VEGF-C. VEGF-C expression lower in mucinous than other subtypes but this was not significant. |
| Ueda et al, 2001 | Expression of VEGF-C mRNA differed markedly among 16 human gynaecological cell lines, and there was correlation between VEGF-C gene expression and the number of cells that migrated and invaded. |
| Ueda et al, 2005 | VEGF-C gene and protein expression differed markedly among 10 ovarian cell lines, |
| Yokoyama et al, 2003 | 66% of 59 EOC expressed VEGF-C. |

**Table 4.14 Studies of VEGF-C in ovarian carcinomas.**

From Table 4.14, two studies reported 66 to 72.5% of the ovarian carcinomas studied to be positive for VEGF-C proteins (Nishida et al, 2004; Yokoyama et al, 2003). In this present study, 84.3% (43/51) of EOC were positive for VEGF-C, but no correlation was found between VEGF-C and histological subtype, stage or grade. This finding agrees with the study of Yokoyama et al, in which they also fail to find a correlation between VEGF-C and histological type, stage, grade, distant metastasis and age at surgery (Yokoyama et al, 2003). In contrast, Ueda et al, using RT-PCR, found a significant correlation of VEGF-C mRNA with stage (Ueda et al, 2005). In addition, Nishida et al, even proposed that VEGF-C was an

independent risk factor for peritoneal and lymph node metastasis (Nishida et al, 2004).

In this present study, like for VEGF, VEGF-C expression was also higher in endometrioid and clear cell EOC subtypes than in serous or mucinous subtypes; finding also reported by Nishida et al (2004), clearly indicating the importance of both angiogenesis and lymphangiogenesis in the malignant transformation of these histological subtypes.

### 4.5.3 VEGFR-1 IN EOC

In the literature, results regarding VEGFR-1 analysis have showed strong gene or protein expression in ovarian malignant tumours (Table 4.15).

| **Authors** | **Results** |
|---|---|
| Abu-Jawdeh et al, 1996 | VEGFR-1 mRNAs were detected in 27 of 29 malignant tumours. |
| Boocock et al, 1995 | VEGFR-1 mRNAs were detected in 3 of 4 ovarian carcinoma cell lines. |
| Chen et al, 2004 | VEGFR-1 protein expression in ovarian carcinomas higher than that in benign and normal ovarian tissues. |
| Inan et al, 2006 | VEGFR-1 immunoreactivity detected in vascular endothelial cells and at malignant sites. |
| Orre and Rogers, 1999 | VEGFR-1 protein expression was lower in mucinous tumours compared to other subtypes. |

**Table 4.15 Studies of VEGFR-1 in ovarian carcinomas.**

Orre and Rogers reported a significant decrease in VEGFR-1 immunostaining in mucinous compared to serous tumours (Orre and Rogers, 1999). Furthermore, Chen et al demonstrated that VEGFR-1 immunoreactivity was stronger in ovarian carcinoma cells than those in both benign and normal ovarian epithelial cells (Chen et al, 2004).

In this study, a higher proportion of EOC samples stained positive for VEGFR-1 (90.5%) but no significant difference was obtained between VEGFR-1 expression and histological type, stage or grade. In contrast to Chen et al's study, this study reported stronger intensity staining of VEGFR-1 in both EOC samples and normal cells. Several reasons may account for this discrepancy. Chen et al used the same polyclonal VEGFR-1 used in this study but at a more diluted concentration (1/150 dilution). Their scoring system was also less simple, being subdivided into four categories depending on the % of cells stained with a higher cut-off threshold. In this study, the approach for VEGFR-1 staining was much better as the VEGFR-1 antibody was more concentrated (1/40 dilution) to reduce the risk of background interference and hence a much simpler scoring system used. Despite these differences in results regarding normal ovaries, EOC tumours, from my study together with Chen et al study (2004) showed high VEGFR-1 expression, suggesting that VEGFR-1 via its binding with VEGF, play a key role in the angiogenic events of EOC progression.

### 4.5.4 TP IN EOC

From Table 4.16, to my knowledge, all the studies found in the literature demonstrate higher TP protein or gene expression in EOC than normal ovarian tissues. In addition, similar results were observed regarding soluble serum TP levels in EOC patients compared to serum from patients with normal ovarian tissues (Watanabe et al, 2003).

| **Authors** | **Results** |
|---|---|
| Fujiwaki et al, 2000 | TP gene expression higher in EOC than normal ovarian tissues. |
| Hata et al, 1999 | TP gene expression was positive in 32% of 38 EOC. |

| | |
|---|---|
| Miszczak-Zaborska et al, 2004 | Higher TP activity in malignant tumours and serum specimens from ovarian cancer patients when compared to control. |
| Nakanishi et al, 1997 | TP is increased in stromal cells of patients with advanced ovarian carcinoma |
| Reynolds et al, 1994 | Significant difference in TP mRNA expression between benign and malignant tumours. |
| Sundar et al, 2006 | Strong staining of TP noted in 1% (epithelial) and 31% (stromal) of 108 tumours. |
| Tanaka et al, 2002 | 78.2% of 101 tumours were positive for TP. |
| Watanabe et al, 2003 | Serum TP levels significantly higher in EOC than controls, but significantly lower in mucinous than other subtypes. |
| Terai et al, 2000 | 23 of 54 ovarian carcinomas were TP positive. |

**Table 4.16 Studies of TP in ovarian carcinomas**

In this study consisting of a small cohort of 26 EOC cases, 76.9% (20/26) of tumours were immunopositive for TP. This result is higher than that obtained in the study by Terai et al, in which out of the 54 EOC cases studied, 42% (23/54) expressed TP (Terai et al, 2000). This difference in result may be explained by the fact that Terai et al (2000) assigned sections to be negative for TP if there are less than 30% of TP positive cells over the total number of cancer cells, whereas in this study the presence of a lesser percentage of TP positive cells (i.e. 10%) were considered negative. Thus using the latter method of scoring, higher proportions of cases are more likely to be scored as positive.

Furthermore, several studies have shown that TP is closely associated with histological EOC features. Reynolds et al, found that TP expression is correlated with the malignant potential of ovarian tumours (Reynolds et al, 1994). Nakanishi et al reported that the immunopositivity of TP in stromal cells is increased in

patients with advanced ovarian carcinoma (Nakanishi et al, 1997). In contrast, this study did not show any apparent correlation between TP positivity and EOC histological features such as subtype or stage. However, in my study, a significant increase in TP expression from grade 2 to 3 was obtained ($p=0.01$) but not between grade 1 to 3, probably due to the small number of grade I cases analysed ($n=1$). This finding agrees with the study of Hata et al in which histological grade was significantly associated with TP gene expression (Hata et al, 1999). Moreover, they reported that FIGO stage, residual disease and histological grade were significantly associated with worse prognosis in univariate analysis. Despite these differences in TP expression and histological features, these results indicate that TP levels may not be reliable at present as a prognostic factor but is an important angiogenic factor in the biology of EOC.

### 4.5.5 MVD IN EOC

Table 4.17 presents the findings of other studies for MVD in EOC, and the differences in antibodies used that may explain for some of the discrepancies among the results. In addition to these studies, this section has analysed MVD in EOC and correlated the results obtained with several angiogenic factors, including a comparison with normal ovaries.

| Author | Antibody | Results |
|---|---|---|
| Abulafia et al, 1997 | vWF | No association between MVD and age, stage, type, CA125, size or patient survival. |
| Abulafia et al, 2000 | vWF | MVD higher in stage I EOC than in stage I borderline tumours. Similar MVD in benign and malignant tumours. |
| Alvarez et al, 1999 | vWF | No association between MVD and age, stage or grade. |

| | | |
|---|---|---|
| Amis et al, 2005 | CD31<br>CD34<br>vWF | MVD higher in functional cysts than in other groups studied.For fixed sections, HVD higher in mucinous than in serous EOC. Similar MVD in benign and malignant tumours. |
| Brustmann et al, 1997 | vWF | MVD higher in EOC than in benign cases ($p<0.001$). |
| Heimburg et al, 1997 | CD34 | No relation between MVD and disease outcome or tumour type. |
| Hollingsworth et al, 1995 | CD34 | Stage as best predictor of overall survival. |
| Karavasilis et al, 2006 | CD34 | No relation between MVD and VEGF. |
| Nakanishi et al, 1997 | vWF | A trend to correlate between MVD and VEGF expression. |
| Obermair et al, 1999 | CD34 | No association between MVD and age, stage, and histological grade. |
| Ogawa et al, 2002 | CD34 | No association between MVD and age, histological type or stage. |
| Orre et al, 1998 | CD31<br>CD34<br>vWF | MVD similar in benign and malignant tumours. |
| Orre and Rogers, 1999 | CD31 | Negative correlation between MVD and VEGFR-1 in benign and serous tumours |
| Raspollini et al, 2004 | CD34 | A trend to correlate between MVD and VEGF expression. |
| Shen et al, 2000 | vWF | No association between MVD and histological parameters. No relation between MVD and VEGF. |
| Sonmezer et al, 2004 | vWF | No correlation between MVD and VEGF. |
| Sundar et al, 2006 | CD31 | No association between MVD and age, stage, grade or residual disease. No correlation between MVD and VEGF or TP. |

**Table 4.17 Other studies and results on MVD.**

From the previous table, Amis et al, showed that MVD is higher in functional cysts than in EOC (Amis et al, 2005) and Shen et al found that MVD values are similar between benign, borderline and malignant ovarian tumours (Shen et al, 2000).

In this present study, MVD was statistically higher in normal ovaries than in EOC cases studied, but AVD values were similar in endometriosis (44.7 ± 23.8), borderline tumours (47.2 ± 21.5), and serous EOC (44.6 ± 17), as shown in Table 3.9 for benign lesions (Chapter 3, section 3.4.5) and 4.15 for EOC (chapter 4 section 4.4.5.1) respectively. These findings are consistent with those of other authors in which MVD are similar in benign and malignant ovarian tumours (Abulafia et al, 1997; Amis et al, 2005; Orre et al, 1998), suggesting that the angiogenic pathways underlying the growth of these tumours may be via a common route, most probably involving VEGF and TP pathways.

Most of the studies on MVD failed to reveal any statistical correlation between MVD and the histological features of EOC (Abulafia et al, 1997; Alvarez et al, 1999; Heimburg et al, 1997; Karavasilis et al, 2006; Obermair et al, 1999; Ogawa et al, 2002; Raspollini et al, 2004; Shen et al, 2000; Sundar et al, 2006). In contrast one study on both fixed and frozen sections, demonstrated that in fixed sections MVD is higher in mucinous than serous EOC subtype; however in frozen sections, MVD did not achieve any statistical significance between the same two tumour types (Amis et al, 2005).

In this study, no correlation was found between MVD and histological EOC subtype but MVD was significantly higher in stage I than that in stage III ($p<0.01$) and higher in grade 2 than in grade 3 ($p=0.03$). On the basis of these differences in results, a potential explanation for these discrepancies may be due to the

heterogeneous pattern of the EOC tumours, hence resulting in MVD values varying from tumour to tumour and also within one and the same tumour. The fact that MVD is higher in stage I and grade 2 tumours and VEGF higher in grade 2 tumours suggest that neovascularisation in EOC may be more intense in the early phase of the development of these tumours, thus more vessels are formed to provide nutrients for the rapid growth of such tumours.

### 4.5.6 DOUBLE MARKER EXPRESSION

In the literature, some studies have attempted to correlate several angiogenic proteins in EOC (Karavasilis et al, 2006; Nakanishi et al, 1997; Orre and Rogers, 1999; Sundar et al, 2006). Karavasilis et al did not find a relationship between VEGF, TSP-1 and MVD in EOC (Karavasilis et al, 2006); however, Nakanishi et al (1997) demonstrated that MVD in VEGF positive and TGF-β positive tumours was significantly higher than that of VEGF-poor and TGF-β negative tumours (Nakanishi et al, 1997). Other authors postulated that VEGF and TP work synergistically in many malignancies (Moghaddam et al, 1995; Ferrara et al, 2004), however, Sundar et al did not find any impact of VEGF and TP expression on survival in ovarian cancer patients (Sundar et al, 2006). Interestingly, this study showed significant correlations when two markers are studied at the same time, i.e. between EOC and controls. These markers are VEGF associated with VEGF-C, VEGFR-1 or TP. The expression of these double markers suggests that these combinations of proteins may work in synergy in ovarian cancer progression and hence may be of diagnostic relevance in EOC.

### 4.5.7 PROTEIN EXPRESSION AND MVD IN EOC

In the literature some authors fail to find a correlation between VEGF immunostaining and MVD (Hartenbach et al, 1997; Karavasilis et al, 2006; Shen et al, 2000; Sonmezer et al, 2004), however, others demonstrated that MVD in VEGF positive was higher than that of VEGF-poor tumours, although this was not significant (Nakanishi et al, 1997; Raspollini et al, 2004). In this study, a good correlation was found between MVD and VEGF positive tumours, in which MVD was significantly higher in VEGF positive tumours than those of VEGF-negative tumours. As a result, the correlation in this study or the trend of correlation shown by some authors (Nakanishi et al, 1997; Raspollini et al, 2004) between MVD and VEGF expression, suggests that an increased in VEGF expression is associated with the growth activity of the tumours cells, which is most probably assisted by the formation of new blood vessel supply to provide nutrients for the growing tumours.

In addition to this study, the expression of other angiogenic factors including VEGF, VEGF-C, VEGFR-1, and TP were also investigated in relation to MVD. Unlike VEGF, these other factors did not show a significant relationship between MVD and the expression of VEGF, VEGF-C, VEGFR-1 or TP; although there was a tendency for MVD to be lower in the positive cases than that in the corresponding negative cases. The fact that MVD is lower in these positive tumours for these growth factors may indicate that VEGF-C, VEGFR-1 or TP do not contribute to the vascularisation of these tumours but may be related to another aspect of tumour growth. The study by Terai et al, is in accordance with this study in which no correlation was found between MVD and TP expression (Terai et al, 2000). However, Hata et al, using ultrasonography reported a

correlation between TP, MVD and peak systolic velocity of the tumour vessels (Hata et al, 1998). The lack of correlation between these proteins and MVD may be due to the numbers of samples that may be too small.

## 4.6 CONCLUDING RESULTS

In summary, the findings from this chapter are as follows:

1. The increase in VEGF expression in serous EOC and mucinous EOC compared to the respective pre-malignant adenoma subtypes suggest this marker is involved in the angiogenic formation of serous and mucinous EOC. Since VEGF does not increase further along EOC stages/grades, the angiogenic process is most likely to take place in the early phase of serous/mucinous tumour formation.
2. As TP expression is also increased alongside VEGF from benign ovarian neoplasms to EOC, TP action may work in parallel with VEGF, implying that TP is as important as VEGF in the early phase of tumour cell progression.
3. The increase in VEGF from mucinous borderline tumours to mucinous EOC reflects the importance of angiogenesis in these tumours to malignant transformation. However, VEGF activity is not paralleled with TP as TP seems to be already involved in the formation of these borderline tumours.
4. In endometriosis, VEGF and TP are highly expressed suggesting that further angiogenic pathways may not be required for the progression towards endometrioid and clear cell EOC.
5. However, the increase in VEGF from endometrioid/clear cell borderline tumours to endometrioid/clear cell EOC indicates the necessity of this

growth factor for the malignant transformation of these tumours and thus reflects that this transformation to malignancy is still angiogenesis-related.

6. Lymphangiogenesis, as measured by VEGF-C protein production, is raised in the pre-malignant lesions but not in EOC, suggesting that it is therefore not required in early EOC formation; however because VEGF-C shows a tendency to increase with high grades, it may be added to a role in lymph node metastasis.

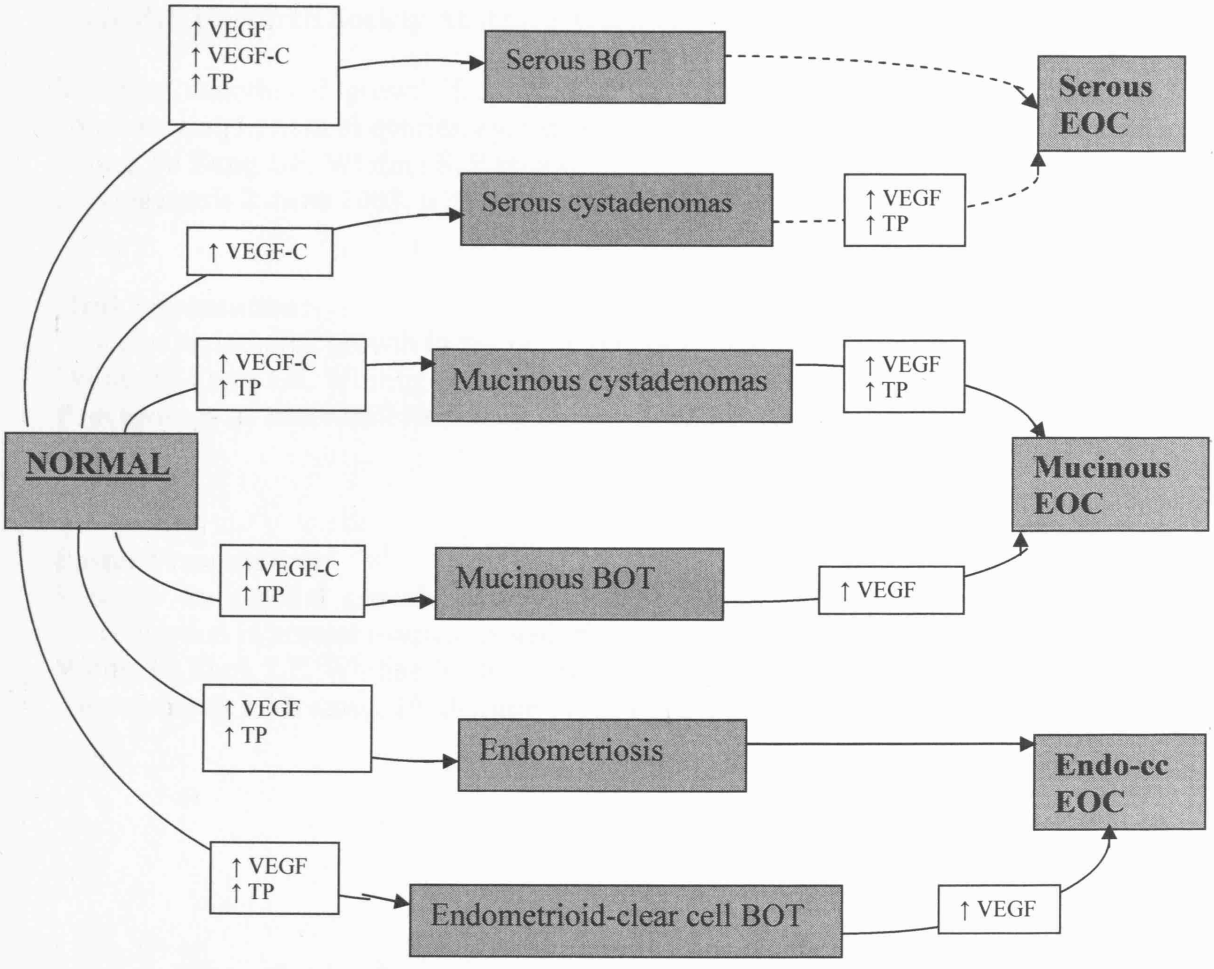



**Figure 4.13 Expression of potential prognostic markers in EOC.**

# CHAPTER 5

# SOLUBLE LEVELS OF VEGF AND VEGFR-2 IN PATIENTS WITH OVARIAN DISEASES

**Abstracts:**
Vascular endothelial growth factor in the sera of patients with ovarian cancer.
**Wong Te Fong LF**, Whiting S, Bamberger ES, MacLean AB, Perrett CW.
**Blair Bell Research Society Abstracts October 2002.**

Vascular endothelial growth factor-A (VEGF-A) tissue expression and serum concentration in normal ovaries, cystadenomas and ovarian cancer.
**Wong Te Fong LF**, Whiting S, Bamberger E, MacLean AB, Perrett, CW.
**Angiogenesis 2 June 2003, p 35-36.**

**Oral Presentation:**
Vascular endothelial growth factor in the sera of patients with ovarian cancer.
**Wong Te Fong LF**, Whiting S, Bamberger ES, MacLean AB, Perrett CW.
**Proceedings of Blair Bell Research Society Meeting, October 2002, Sheffield UK.**

**Poster Presentation:**
Vascular endothelial growth factor-A (VEGF-A) tissue expression and serum concentration in normal ovaries, cystadenomas and ovarian cancer.
**Wong Te Fong LF**, Whiting S, Bamberger E, MacLean AB, Perrett, CW.
**Angiogenesis 2 Meeting, 19-20 June 2003, Paris France.**

## 5.1 INTRODUCTION

During the last decade, huge efforts have been concentrated towards improving outcomes for ovarian cancer by screening for pre-clinical, early stage disease using both imaging techniques and serum markers. To date, the most thoroughly investigated biomarker in ovarian cancer screening is CA-125.

In the United Kingdom, a large randomised controlled trial (RCT) of ovarian cancer screening (OCS) is currently being conducted involving 200,000 postmenopausal women (UK Collaborative trial of Ovarian Cancer Screening, UKCTOCS, www.ukctocs.org). This trial incorporates a novel screening strategy utilising CA-125 and ultrasound developed during the last decade (Menon et al, 2005). It was demonstrated that a multimodal screening strategy to detect ovarian cancer, using serum CA-125 as the first line test and pelvic ultrasound as the secondary test can achieve high specificity (99.9%) and positive predictive value of ovarian cancer screening (Jacobs et al, 1988; 1993). In the RCT that followed, median survival was significantly increased in women with ovarian cancer in the screened group (72.9 months) when compared with the control group (41.8 months; Jacobs et al, 1999). Recently, attempts have been made to quantify CA-125 in combination with other serum and urine markers such as oncofetal antigens, mucin-like proteins and co-enzymes in ovarian cancers (Bast et al, 2002).

In healthy adults, VEGF is a key regulator of angiogenesis that increases microvascular permeability (Neufeld et al, 1999). It acts via two tyrosine-kinase receptors, VEGFR-1 and VEGFR-2. Biological responses mediated by the activation of these two receptors are somewhat different; activation of VEGFR-2 induces cell proliferation and facilitates changes in the extracellular matrix that

are needed to permit new vessel formation (Giudice, 1996), while the activation of VEGFR-1 does not (Neufeld et al, 1999). In addition, VEGFR-2 appears to play a more functional role in mediating signalling events involved in endothelial cell mitogenesis, migration, survival and vascular permeability (Shibuya, 2001).

As VEGF is known to increase vascular permeability, when substantial amounts of VEGF are present in cysts fluid (Abu-Jawdeh et al, 1996) or ascitic fluid (Yeo et al, 1993), VEGF itself may be released into the patient's serum. However, some authors have doubted the validity of measuring serum levels of VEGF, owing to the ability of some haematological cells to release VEGF (Banks, 1998). The detection of VEGF protein in thrombin-treated platelet suspensions and the presence of VEGF mRNA and protein within human megakaryocytes has provided strong evidence that VEGF synthesis during thrombopoiesis is the origin of platelet VEGF. However, the extent to which these haematological cells normally contribute to the circulating VEGF pool in vivo and the physiological relevance of this is not yet known.

Elevated serum VEGF levels have been detected in a variety of cancers (Ferrara, 1995; Yamamoto et al, 1996; Salven et al, 1997), including colorectal carcinoma (Kumar et al, 1998; George et al, 2000) and vulval cancer (Hefler et al, 1999). Lebrecht et al (2002) reported that the serum VEGF level has been regarded as a surrogate marker of tumour angiogenesis in patients with cervical carcinoma.

To date, controversial data still exist on the detectability of VEGF in patient sera and effusions in cancer patients (Yeo et al, 1993; Kondo et al, 1994) and on the clinical usefulness of serum VEGF, partly due to the influence of platelets during the clotting process. In ovarian cancer, there are a few relevant studies regarding the serum level of VEGF (sVEGF); however, none exists concerning sVEGFR-2.

## 5.2 AIMS

The aims of this chapter were:

- To analyse the fluctuations of sVEGF and sVEGFR-2 levels in healthy women, (serum specimens from women who were still menstruating) and to investigate whether physiological angiogenesis also affects serum concentrations of these factors.
- To investigate soluble sVEGF and sVEGFR-2 in serum and malignant effusions collected from patients with various subtypes of EOC or patients with benign ovarian cystadenomas compared with controls (serum specimens from women with no ovarian diseases).
- To look for a correlation between sVEGF and sVEGFR-2 levels, tumour type and disease stage or grade as well as the potential value of these levels as diagnostic markers in EOC.

## 5.3 MATERIALS AND METHODS

Patient recruitment, serum sample and fluid collection from ovarian cysts were performed within the guidelines of protocols approved by the RF Hampstead NHS Trust LREC. Informed consent was obtained from all subjects.

### 5.3.1 HEALTHY WOMEN

Forty-four healthy pre-menopausal women volunteers, still menstruating and having not undergone any major surgery nor having any ovarian diseases, were recruited in this study. They are women whose blood samples were taken on a weekly schedule during the menstrual cycle and immediately processed to separate the serum which was then stored at -80°C. From the 44 volunteers

starting the trial, 29 continued to give blood until week 2 of their menstrual cycle, 21 until week 3 and only 13 finished the study. No mean values for age were calculated as some declined to give their date of birth.

### 5.3.2 PATIENTS

#### 5.3.2.1 SERUM SAMPLES

Blood was drawn at pre-operative visit from a total of 90 female patients who underwent elective surgery in 1995-2001 at the Royal Free Hospital for operations including hysterectomy, bilateral salpingo-oophorectomy and omentectomy. The blood samples were then processed and the serum supernatants were stored at -80°C until examination. The list of patients includes 35 patients with EOC, 29 with benign ovarian cystadenomas and 25 women who underwent oophorectomy but had normal ovaries, referred as the control group. The age and tumour characteristics of these patients are listed in Table 5.1.

| **Diagnosis** | **n** | **Mean age ± SD (range), yrs** |
|---|---|---|
| **Healthy individuals** (still menstruating) | 44 | (25-40) |
| **Controls** | 25 | 49.6 ± 11.1 (25-81) |
| **Benign cystadenomas** | 29 | 44.7 ± 12.1 (24-75) |
| **Subtype** | | |
| Serous | 7 | 42.6 ± 10.1 (31-61) |
| Mucinous | 16 | 46.2 ± 13.8 (24-75) |
| Unclassified | 6 | 43.2 ± 9.3 (32-54) |
| **EOC** | 35 | 56.1 ± 14.3 (24-86) |
| **Subtype** | | |
| Serous | 17 | 56.3 ± 11.6 (29-79) |
| Mucinous | 10 | 54.8 ± 18.3 (25-86) |
| Endometrioid and clear cell | 4 | 63.5 ± 18.8 (42-83) |
| Mixed | 2 | 46.5 ± 2.1 (45-48) |

| | | |
|---|---|---|
| Unclassified | 2 | unreported |
| **Stage** | | |
| I | 2 | 50.0 ± 35.4 (25-75) |
| II | 4 | 57.0 ± 21.4 (35-83) |
| III | 23 | 57.0 ± 13.0 (29-86) |
| IV | 1 | 61 |
| **Grade** | | |
| 1 | 3 | 53.0 ± 5.3 (46-57) |
| 2 | 5 | 64.6 ± 14.3 (43-79) |
| 3 | 16 | 57.1 ± 11.2 (36-83) |

**Table 5.1 Tumour characteristics.**

To correlate sVEGF and sVEGFR-2 of these ovarian samples with age, these women were subdivided into two groups and are as follows: women aged 51 or less were characterised as younger women and women aged more than 51 as older women.

5.3.2.2 CYSTIC AND ASCITIC FLUIDS

Ovarian cystic fluids from 24 patients with benign cystadenomas, including 5 patients with serous subtype, 4 with mucinous subtype and ascitic fluids from 9 EOC patients were also available for this study. The mean ± SD age of the 24 patients with benign cystadenomas presenting with cystic fluids was 52 ± 16.7 years and the mean ± SD age of the 9 EOC patients having ascites was 64 ± 18.5 years.

| **Diagnosis** | **n** |
|---|---|
| **Cystic fluids** | 24 |
| Serous cysts | 5 |
| Mucinous cysts | 4 |

| | |
|---|---|
| Unspecified | 15 |
| **Ascitic fluids** | 9 |
| Serous adenocarcinoma | 4 |
| Mucinous adenocarcinoma | 2 |
| Unspecified adenocarcinomas | 3 |

**Table 5.2 Ovarian fluids.**

### 5.3.3 SAMPLE PROCESSING

Peripheral venous blood and other fluids collected for this study, as summarised in Chapter 2, Section 2.1.2.3, were removed from the freezer at -80°C and left on ice to defrost. Samples were then vortexed gently before analysis by ELISA.

### 5.3.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The serum and tissue fluid levels were then analysed for VEGF and VEGFR-2, using commercially available kits, as described in Chapter 2, Section 2.2.2. Each specimen was run in duplicate and the results were averaged.

### 5.3.5 STATISTICAL ANALYSIS

Age was given in mean ± SD and range values. Because of their skewed distribution, median and range values are given to describe VEGF, VEGFR-2 and platelet counts. Also, the $5^{th}$ and $95^{th}$ percentile values were chosen for data description in box plots presentation. The Mann Whitney U test and the Kruskal-Wallis test were used to compare the VEGF or VEGFR-2 distributions across subgroups of patients based on age, diagnosis, grade and stage. Correlations between different parameters were investigated by Spearman's rank correlation coefficient. For example, the correlations between the levels of VEGF and

VEGFR-2 in cystic/ascitic fluid and serum were calculated by Spearman test. Also, the Spearman Correlation Coefficient was used to determine if there was a correlation between VEGF, VEGFR-2, and platelet counts. Statistical significance was set at $p<0.05$.

## 5.4 RESULTS

The mean ± SD (range) ages of the different groups were calculated and are as follows: the control group was 49.6 ± 11.1 (25-81), benign group 44.7 ± 12.1 (24-75), malignant group 56.1 ± 14.3 (24-86) years (Table 5.1). Because the means of these three age groups had such a wide standard deviation, the differences were not significant.

### 5.4.1 SERUM VEGF LEVELS (sVEGF)

#### 5.4.1.1 sVEGF AND HEALTHY VOLUNTEERS

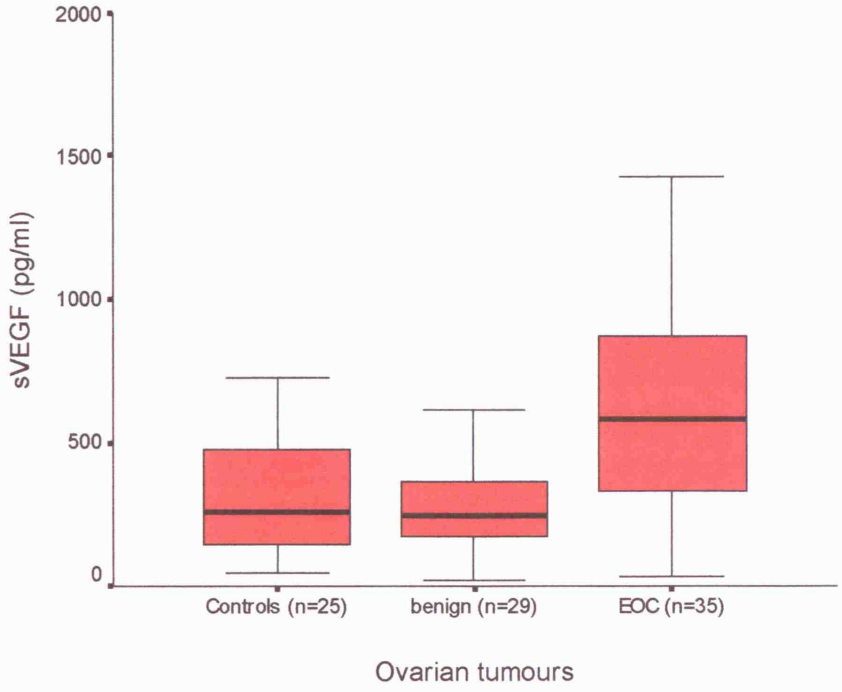
Descriptive statistics for sVEGF levels subdivided by the time when serum samples were taken from these women are shown in Table 5.3. Overall, the median and range sVEGF for all 4 weeks were 330 and 52.7-1779.2 pg/ml. There was no significant difference between the length of the menstrual cycle and sVEGF ($p= 0.26$, $\chi 2$ test).

| Group | sVEGF levels (pg/ml) | | |
|---|---|---|---|
| | n | Median | Range |
| All | 44 | 330 | 52.7-1779.2 |
| 1st week of cycle | 44 | 359 | 57-1779 |
| 2nd week of cycle | 29 | 356 | 60-1430 |
| 3rd week of cycle | 21 | 304 | 53-1021 |
| 4th week of cycle | 13 | 266 | 92-1121 |

**Table 5.3 Comparison of serum VEGF levels in pre-menopausal women.**

5.4.1.2 sVEGF AND PATIENTS WITH OVARIAN DISEASES

From Figure 5.1, the median (range) of sVEGF were 257 (48-1425) pg/ml for the controls, 248 (18-1128) pg/ml for cystadenomas and 598 (30-2110) pg/ml for EOC.




The upper and lower quartiles and the median values are depicted as box plots. Whiskers indicate the 5th and 95th percentiles.
**Figure 5.1 sVEGF in ovarian tissues.**

Table 5.4 shows statistical comparisons between sVEGF levels and these ovarian tumours, revealing that sVEGF was significantly higher in EOC patients than in controls or benign cystadenomas patients, using the Mann Whitney U test.

| Diagnosis | Controls | Benign cystadenomas |
|---|---|---|
| **Benign cystadenomas** | 0.75 | |
| **EOC** | **0.001** | **0.0001** |

**Table 5.4 P values for sVEGF between controls and ovarian diseased patients.**

5.4.1.3 sVEGF AND BENIGN CYSTADENOMA SUBTYPE

When these ovarian cystadenomas were subdivided according to the histological type, the median (range) sVEGF resulting for the different subtypes were; serous = 218 (46-520), mucinous = 231 (78-1128) and other unclassified cysts = 317 (18-359) pg/ml (see Figure 5.2).

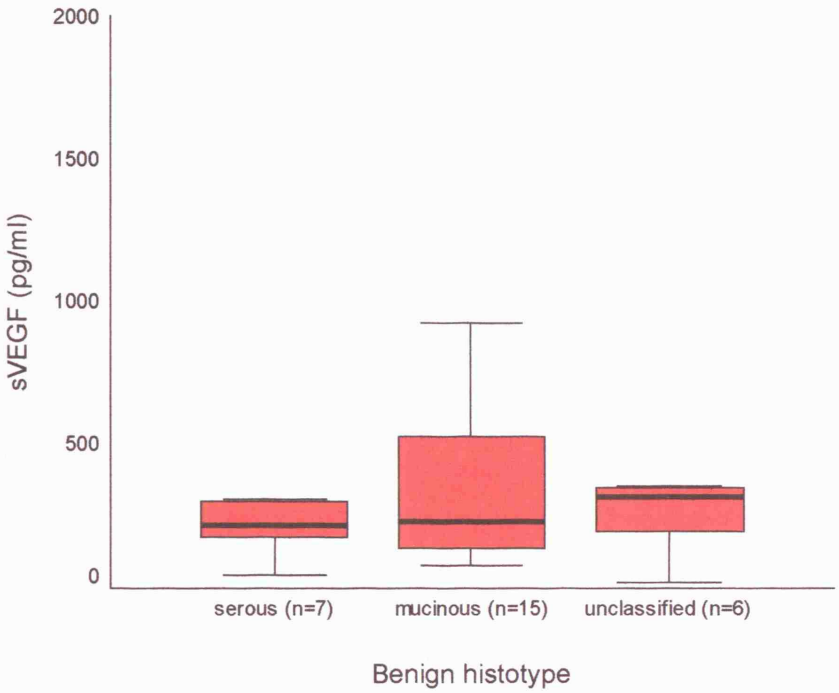



**Figure 5.2 sVEGF in benign cystadenomas according to histological subtype.**

Statistical analysis comparing sVEGF between each benign histological subtype showed no significant difference between sVEGF and benign subtypes (Table 5.5).

| Subtype | Serous | Mucinous |
|---|---|---|
| **Mucinous** | 0.78 | |
| **Unclassified cyst** | 0.63 | 0.97 |

**Table 5.5 P values for sVEGF between benign cystadenoma histological subtypes.**

5.4.1.4 sVEGF AND EOC

Among patients with EOC, the overall median and range values for sVEGF levels were 598 and 30-2110 pg/ml. Figure 5.3 shows the median (range) values for sVEGF levels in patients aged less than 51 years old were significantly lower than those in patients aged more than 51 (age ≤ 51: 386 (207-793) pg/ml, age >51: 745 (range 30-2110) pg/ml respectively, with p=0.02, using the Mann Whitney U test.

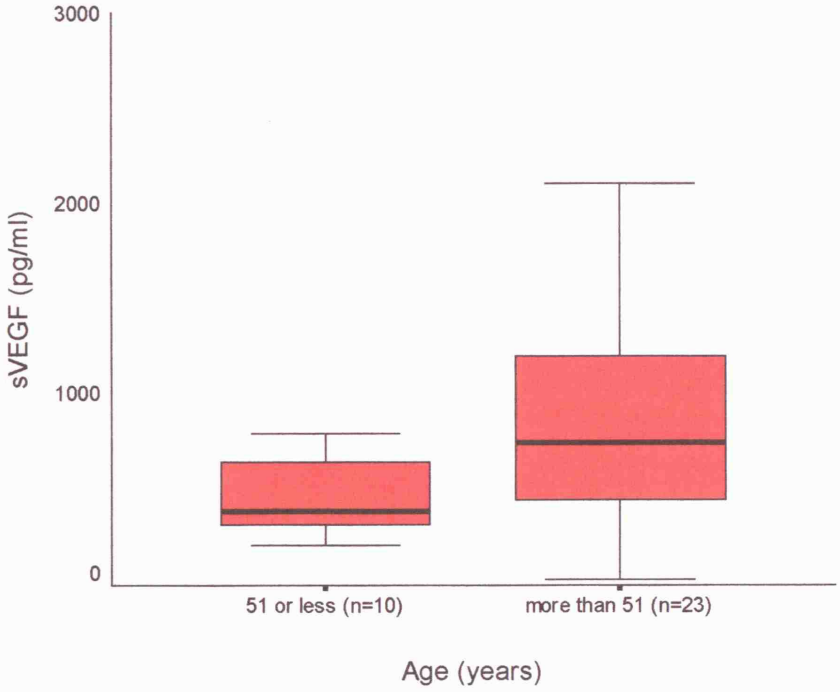



**Figure 5.3 sVEGF in EOC women aged ≤51 or >51.**

5.4.1.5 sVEGF AND EOC HISTOLOGICAL SUBTYPE

Comparisons between median (range) sVEGF and each EOC histological subtype were analysed and are as follows: serous = 622 (30-1961), mucinous = 531 (207-1176), endometrioid = 779 (313.2-2109.7) pg/ml, and clear-cell = 813, and unclassified = 225.5 (82.1-368.8) pg/ml.

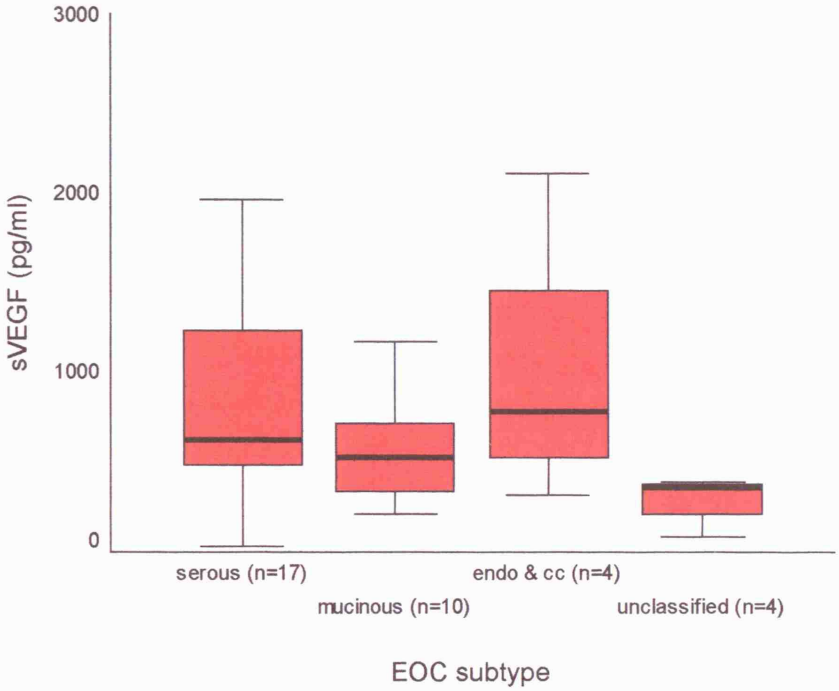



**Figure 5.4 sVEGF in EOC histological subtypes.**

Table 5.6 shows the statistical comparisons between sVEGF and EOC histological subtype, revealing no significant difference between sVEGF and EOC subtype.

| Histological subtype | Serous | Mucinous | Endometrioid & clear cell |
|---|---|---|---|
| **Mucinous** | 0.17 | | |
| **Endometrioid & clear cell** | 0.65 | 0.14 | |
| **Unclassified** | 0.08 | 0.11 | 0.13 |

**Table 5.6 P values for sVEGF between EOC histological subtypes.**

5.4.1.6 sVEGF AND EOC STAGE

Figure 5.5 shows the median (range) of sVEGF between EOC stages and the results are as follows: stage I = 272 (207-338), II = 584 (378-2110), III= 622 (30-1961), IV = 714 pg/ml and unclassified = 369 (82-771). Again for statistical reasons, due to the one sample of stage IV in this study, this sample was analysed with samples whose stages were not reported.

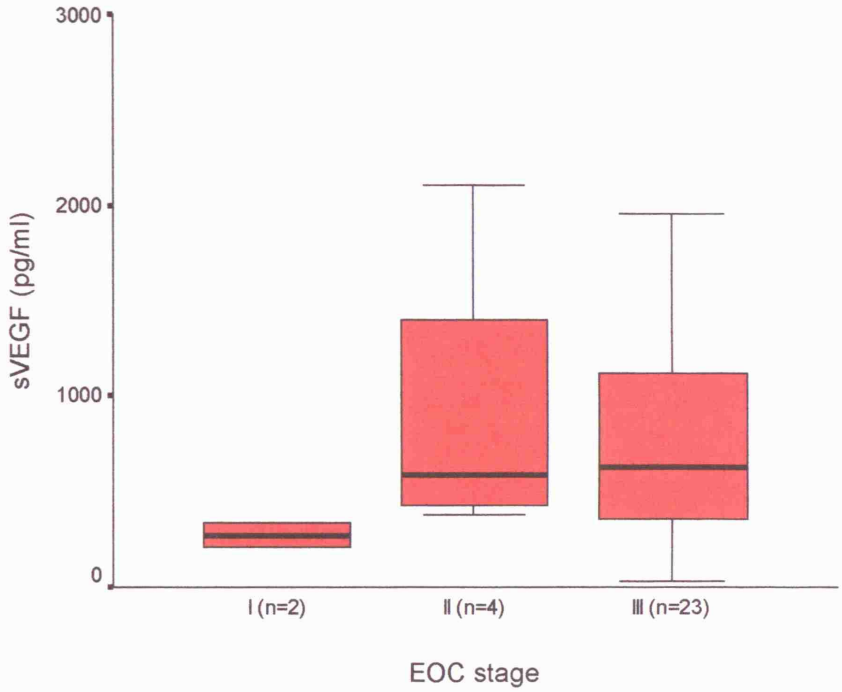



**Figure 5.5 sVEGF in EOC tumour stages.**

The results of the statistical analysis between sVEGF and tumour stage are shown in Table 5.7, demonstrating no relationship between sVEGF and tumour stage.

| Tumour stage | I | II |
|---|---|---|
| II | 0.35 | |
| III | 0.19 | 0.68 |

**Table 5.7 P values for sVEGF between EOC tumour stages.**

5.4.1.7 sVEGF AND EOC DISEASE GRADE

From Figure 5.6, the median (range) of sVEGF values for grade 1 was 225 (30-771), grade 2 was 813 (278-1277), and grade 3 was 670 (313-2110).

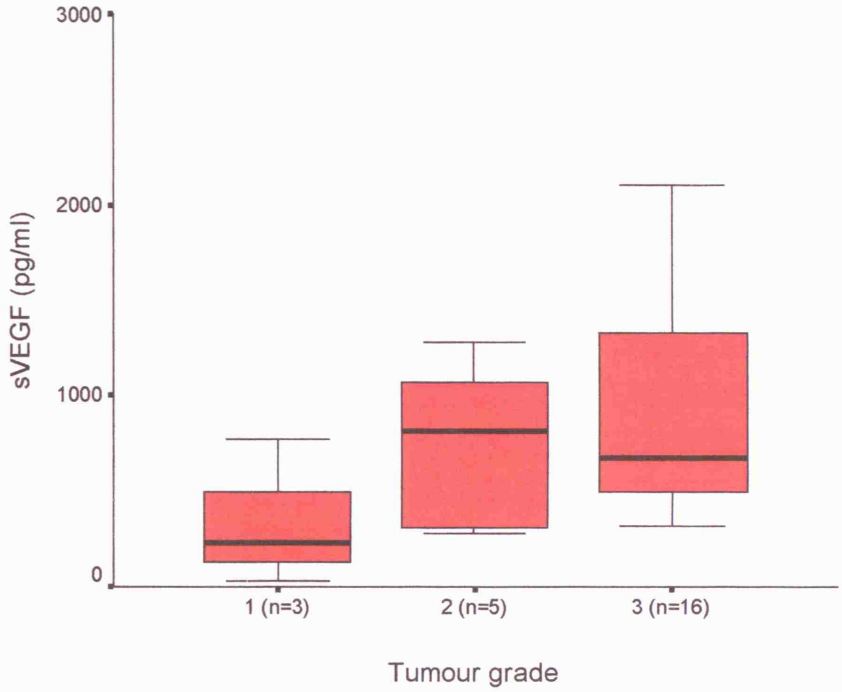



**Figure 5.6 sVEGF in EOC tumour grades.**

Statistical analysis as shown in Table 5.8 showed no significant difference between sVEGF and tumour grades.

| Disease grade | 1 | 2 |
|---|---|---|
| 2 | 0.24 | |
| 3 | 0.13 | 0.54 |

**Table 5.8 P values for sVEGF between EOC grades.**

### 5.4.2 SERUM VEGFR-2 LEVELS (sVEGFR-2)

5.4.2.1 sVEGFR-2 AND HEALTHY VOLUNTEERS

Due to the insufficient amount of serum available, sVEGFR-2 concentrations were only measured on 8 patients during their 1st week of menstrual cycle, 8 patients during their 2nd week, 4 during their 3rd week and 2 during their last week of the menstrual cycle.

The overall median sVEGFR-2 for all weeks combined was 9608 pg/ml (range 7290-12072). Table 5.9 summarises the descriptive statistics of sVEGFR-2 levels for each week of the menstrual cycle. No correlation was found between the length of the menstrual cycle and sVEGFR-2 (P=0.624).

| **Group** | **sVEGFR-2 levels in pg/ml** | | |
|---|---|---|---|
| | ***n*** | **Median** | **Range** |
| **1st week of cycle** | 8 | 9715 | 7343-12072 |
| **2nd week of cycle** | 8 | 9564 | 8079-11706 |
| **3rd week of cycle** | 4 | 8913 | 7290-11754 |
| **4th week of cycle** | 2 | 9292 | 8447-10087 |

**Table 5.9 Comparison of serum VEGFR-2 levels in pre-menopausal women.**

5.4.2.2 sVEGFR-2 AND PATIENTS WITH OVARIAN DISEASES

The median (range) of sVEGFR-2 was 8287 (4547-12123) pg/ml for controls, 8159 (4308-15007) for cystadenomas and 7922 (1267-13319) pg/ml for EOC (Figure 5.7).

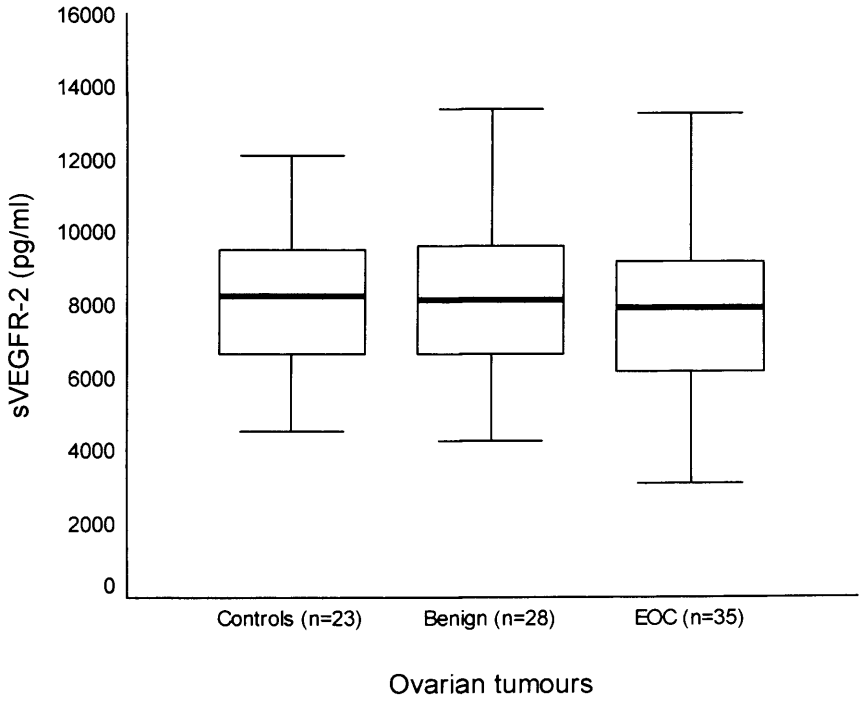



**Figure 5.7 sVEGFR-2 in ovarian tumours.**

As demonstrated in Table 5.10, statistical analysis did not show any correlation between sVEGFR-2 levels and these ovarian tumours.

| Diagnosis | Controls | Benign cystadenomas |
|---|---|---|
| **Benign cystadenomas** | 0.76 | |
| **EOC** | 0.35 | 0.20 |

**Table 5.10 P values for sVEGFR-2 between controls and ovarian tumours.**

5.4.2.3 sVEGFR-2 AND BENIGN CYSTADENOMA SUBTYPES

As shown in Figure 5.8, the median and range sVEGFR-2 values were also subdivided according to histological subtypes of benign cystadenomas and are as follows: serous was 7604 and 5735-11492 pg/ml and mucinous was 8215 and 5687-15007 pg/ml. Statistical analysis comparing sVEGFR-2 between these two subtypes did not reach significant difference (p=0.54).

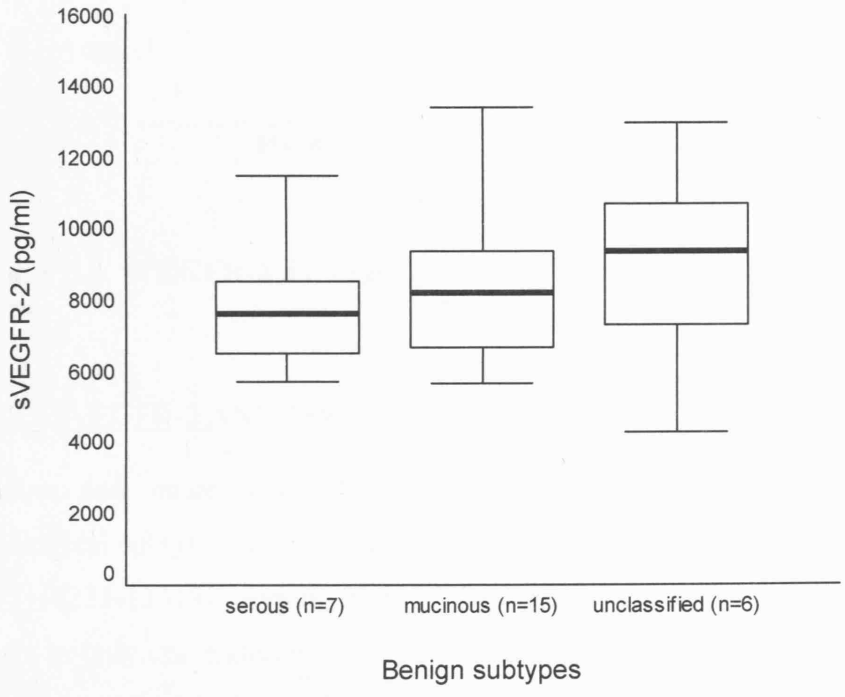



**Figure 5.8 sVEGFR-2 in benign ovarian cystadenoma histological subtypes.**

5.4.2.4 sVEGFR-2 AND EOC

In EOC, the overall median and range values for sVEGFR-2 levels were 7922 and 1267-13319 pg/ml. Figure 5.9 shows patients aged 51 or less had lower VEGFR-2 serum levels (median 7763 and range 5064-13318) than those aged more than 51 years (7946 and 3132-12980 pg/ml respectively), although this difference was not statistically significant (p=0.62).

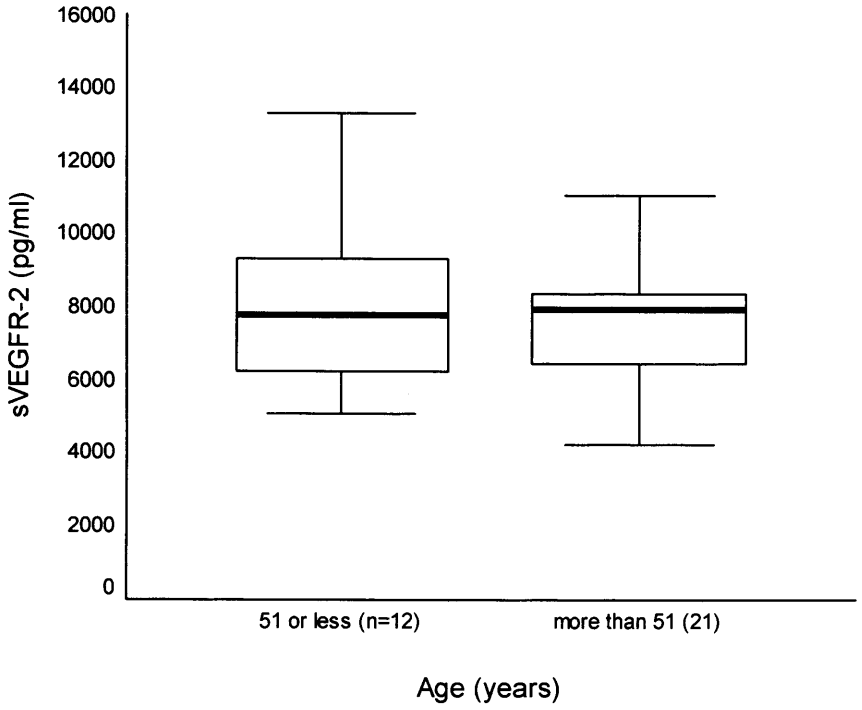



**Figure 5.9 sVEGFR-2 in EOC patients between women aged ≤51 or >51 years.**

5.4.2.5 sVEGFR-2 AND EOC HISTOLOGICAL SUBTYPES

Median and range sVEGFR-2 values were subdivided according to EOC histological subtype and are as follows: serous = 7973 (3123-11078), mucinous = 7171 (4231-13319), endometrioid = 5400 and clear cell = 9147 (7058-9285). Again as only one endometroid sample was available, its median and range values were analysed with clear-cell EOC subtype and the new values were 8102 (5400-9285) pg/ml (Figure 5.10).

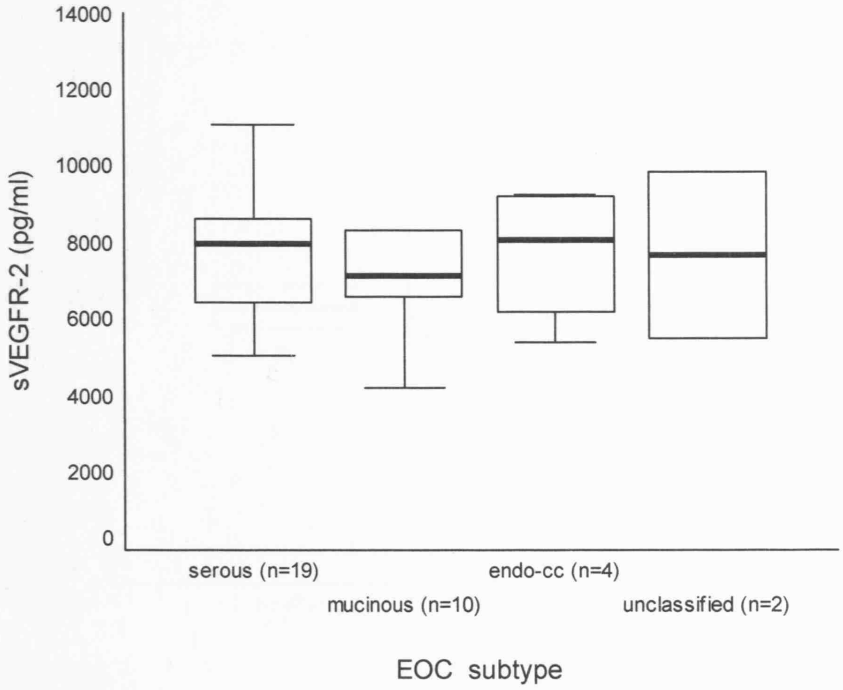



**Figure 5.10 sVEGFR-2 in EOC histological subtypes.**

Table 5.11 shows the statistical comparisons between sVEGFR-2 and EOC histological subtype, demonstrating no significant difference between sVEGFR-2 and EOC subtype.

| Histological subtype | Serous | Mucinous | Endometrioid & clear cell |
|---|---|---|---|
| **Mucinous** | 0.60 | | |
| **Endometrioid & clear cell** | 0.89 | 0.83 | |
| **Others** | 0.68 | 0.62 | 0.78 |

**Table 5.11 P values for sVEGFR-2 between EOC histological subtypes.**

5.4.2.6 sVEGFR-2 AND EOC STAGE

Figure 5.11 shows the median (range) of sVEGFR-2 between EOC stages and the results are as follows: stage I = 7758 (7171-8345), II = 7478 (6779-13319) and III = 7789 (3133-12981). The remaining samples of EOC with unknown stage were grouped together and sVEGFR-2 values were 9285 (1267-11358) pg/ml (results not shown in Figure 5.11).

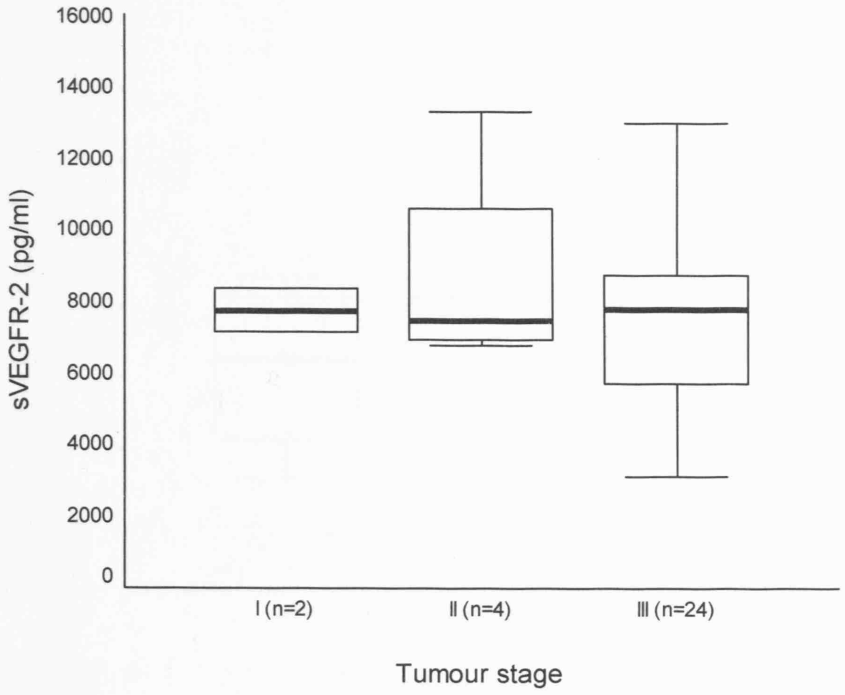



**Figure 5.11 sVEGFR-2 in EOC between tumour stages.**

The p values of the statistical analysis between sVEGFR-2 and tumour stage are shown in Table 5.12, demonstrating no correlation between sVEGFR-2 and tumour stage.

| **Tumour stage** | **I** | **II** |
|---|---|---|
| **II** | 0.69 | |
| **III** | 0.88 | 0.34 |

**Table 5.12 P values for sVEGFR-2 between EOC tumour stages.**

5.4.2.7 sVEGFR-2 AND EOC GRADE

From Figure 5.12, the median (range) of sVEGFR-2 values for grade 1 was 6219 (3133-8800), grade 2 was 8210 (5400-10339), and grade 3 was 7039 (5064-12981).

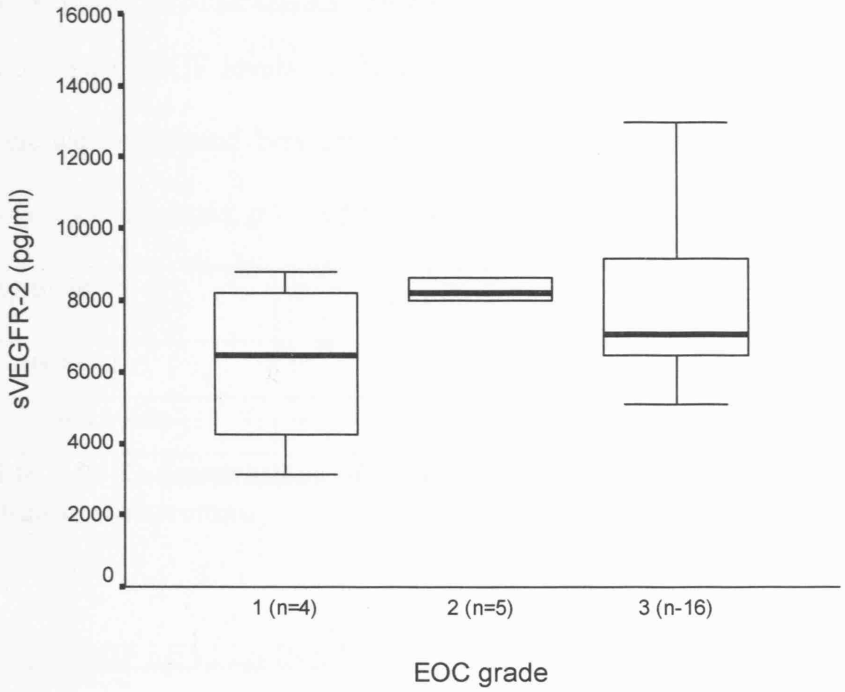



**Figure 5.12 sVEGFR-2 in EOC between tumour grades.**

Statistical analysis as shown in Table 5.13 revealed no significant difference between sVEGFR-2 and tumour grade.

| Disease grade | 1 | 2 |
|---|---|---|
| 2 | 0.23 | |
| 3 | 0.23 | 0.80 |

**Table 5.13 P values for sVEGFR-2 between EOC grades.**

### 5.4.3 OVARIAN FLUIDS

Due to an insufficient amount of the fluids available, only VEGF concentrations were measured in both ovarian cystic and ascitic fluids.

5.4.3.1 VEGF IN CYSTIC FLUIDS OF BENIGN CYSTADENOMAS

The median VEGF levels of these cystic fluids were 966 pg/ml. No significant difference was found between cystic VEGF levels and histological subtype in benign cystadenomas, $p > 0.05$ (Table 5.14)

| **Diagnosis** | **n** | **median** | **range** |
|---|---|---|---|
| **Serous cysts** | 5 | 2509 | 81-5114 |
| **Mucinous cysts** | 4 | 1098 | 103-7550 |

**Table 5.14 Concentrations of VEGF in cystic fluids of different subtypes in benign cystadenomas.**

5.4.3.2 VEGF LEVELS IN CYSTIC FLUIDS AND SERUM OF BENIGN CYSTADENOMAS

Only 4 patients with benign ovarian cystadenomas have matched samples of cystic fluids and serum samples, as shown in Table 5.15 Due to the small number of samples, and the wide range of sVEGF values, statistical analysis could not be performed between sVEGF and these two different fluids.

| **Patient** | **Cystic fluid** | **Serum** |
|---|---|---|
| **1** | 191 | 45.8 |
| **2** | 9.2 | 351.9 |
| **3** | 67.2 | 344.7 |
| **4** | 102.8 | 1127.8 |

**Table 5.15 VEGF levels in cystic fluids and matched serum samples of 4 benign cystadenomas.**

However, when comparing VEGF levels between unmatched cystic fluids and serum samples, cystic fluids showed an average of 4 fold increase than serum

samples, being statistically significant with a p value of 0.009; median values for cystic fluids = 966 pg/ml, and for serum = 248 pg/ml (Figure 5.13).

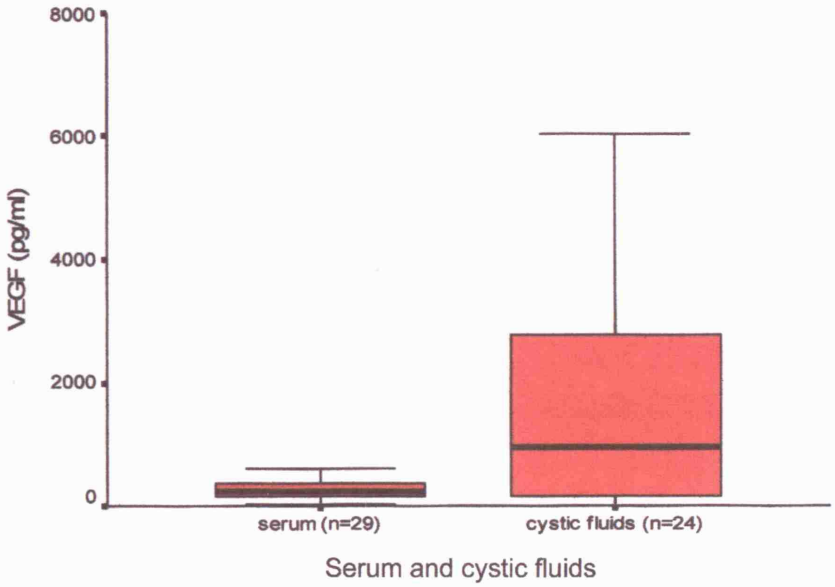



**Figure 5.13 VEGF levels between unmatched serum samples and cystic fluids.**

5.4.3.3 VEGF LEVELS IN EOC ASCITIC FLUIDS

The median (range) of VEGF concentrations in EOC ascitic fluids was 3250 (310.7-5106.1) pg/ml. Because of the small numbers studied, it was difficult to correlate VEGF ascitic levels with other variables available (see Table 5.16).

| Patient | Diagnosis | Grade | sVEGF (pg/ml) |
|---|---|---|---|
| **1** | Papillary adenocarcinoma | 3 | 1162 |
| **2** | Mucinous adenocarcinoma | 1 | 3954 |
| **3** | Serous adenocarcinoma | 3 | 5106 |
| **4** | Adenocarcinoma | 3 | 3250 |
| **5** | Adenocarcinoma | | 1011 |
| **6** | Adenocarcinoma | | 3753 |
| **7** | Serous adenocarcinoma | 3 | 4629 |
| **8** | Serous adenocarcinoma | 3 | 2665 |
| **9** | Mucinous adenocarcinoma | 2 | 310 |

**Table 5.16 Characteristics of 9 EOC patients with ascites.**

5.4.3.4 COMPARISON OF VEGF LEVELS IN SERUM AND ASCITIC FLUIDS

Table 5.17 shows only 3 EOC cases with matched ascitic and serum samples in this study. Again, because of the small numbers, no correlation for VEGF could be performed between ascitic and matched serum samples.

| Diagnosis | VEGF in ascitic fluids | VEGF in serum |
|---|---|---|
| Adenocarcinoma | 3250 | 1433 |
| Adenocarcinoma | 1011 | 483 |
| Serous adenocarcinoma | 2665 | 1961 |

**Table 5.17 VEGF levels in ascitic and serum samples of 3 EOC patients.**

But when comparing unmatched EOC samples of ascitic fluids with serum samples, ascitic fluids (median = 3250 pg/ml) showed an average of 2.3-fold increase than serum samples (median = 1433pg/ml) (Figure 5.14), being statistically significant ($P < 0.0001$, Mann Whitney U test).

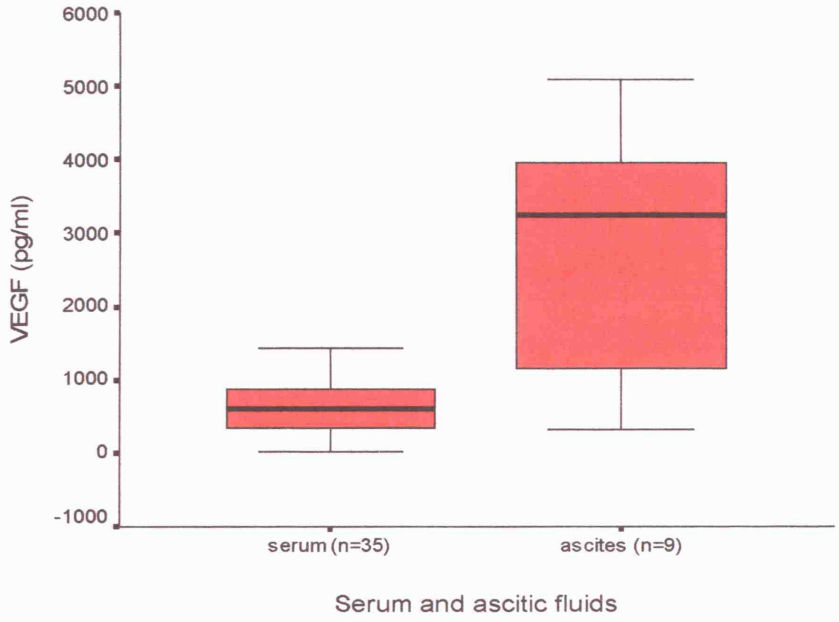



**Figure 5.14 VEGF levels between serum samples and ascitic fluids, ($p < 0.0001$).**

### 5.4.4 PLATELET COUNTS

Of these pre-operative serum samples, platelet counts were also analysed in benign and malignant ovarian tissues and the results are as follows: 258.5 (179-518) in controls, 254 (14-357) in benign cystadenomas, and 316.5 (234-476) in EOC (Figure 5.15). Statistical analysis showed that there was a significant difference in platelet count between benign tumours and EOC, with p=0.03.

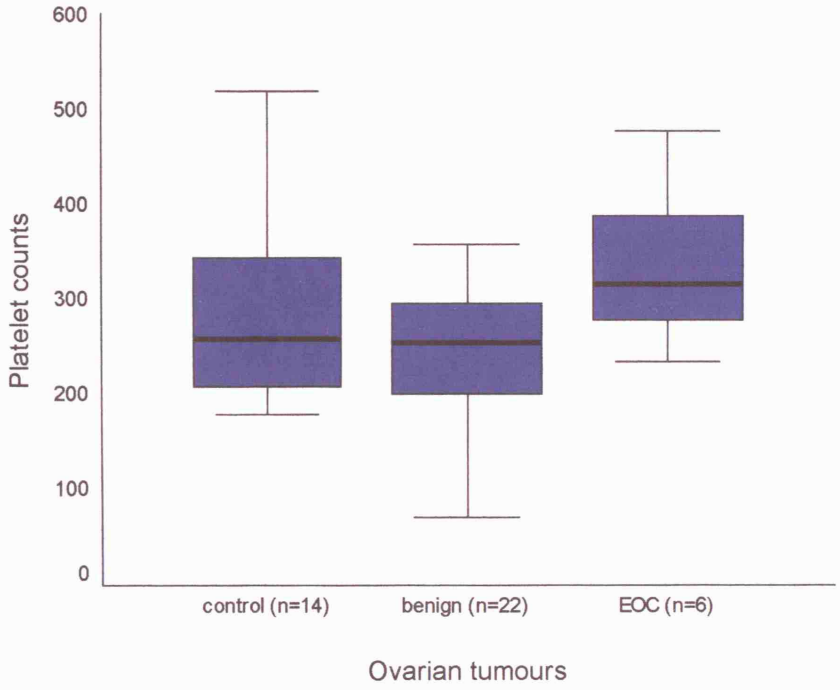



**Figure 5.15 Platelet counts in controls, benign ovarian tumours and in EOC.**

5.4.4.1 VEGF AND PLATELET COUNTS BETWEEN OVARIAN TUMOURS

With regard to a correlation between sVEGF and platelet counts, sVEGF and platelets counts showed an increase from benign to malignant tumours, however, this difference was not statistically significant (p=0.68, see Table 5.18).

| | **Controls** | **Benign** | **EOC** |
|---|---|---|---|
| **sVEGF** | 257 | 248 | 598 |
| **Platelet counts** | 289 | 241 | 335 |
| **p value** | 0.95 | 0.12 | 0.68 |

**Table 5.18 Association between sVEGF and platelet counts between ovarian samples.**

## 5.5 DISCUSSION

### 5.5.1 sVEGF AND HEALTHY VOLUNTEERS

In this study, detectable and similar concentrations of sVEGF were found in the serum of healthy volunteers of blood samples with no significant difference between sVEGF concentrations and the different weeks of the menstrual cycle. However, there was a tendency for elevated sVEGF values in the first two weeks of the cycle (early proliferative phase) versus the last two weeks (secretory phase). These results are in agreement with Benoy et al, who examined sVEGF during the different phases of the menstrual cycle of 6 women (Benoy et al, 1998). They found a higher proportion of samples with elevated sVEGF from early proliferative phase to secretory phase and finally to peri-ovulatory phase. However, a recent study on 30 healthy Swedish women with regular menstrual cycles, reported a profound increase of VEGF starting at the early secretory phase (Malamitsi- Puchner et al, 2004). These discrepancies in results may be due to the fact that our sample number was not consistent during the different time series that VEGF was measured and hence this study did not show any increase at the early secretory phase as observed by Malamitsi- Puchner et al (2004); however these findings although conflicting in values during the different phases of the menstrual cycle show that the amount of circulating VEGF in healthy pre-menopausal women fluctuates during the physiological processes related to the menstrual cycle. The latter suggests that VEGF plays an important role in

endometrial angiogenesis, in hormone-dependent vascular growth, and in the increase of micro-vascular permeability associated with endometrial repair.

### 5.5.2 sVEGF AND OVARIAN DISEASES

Table 5.19 shows the results of other authors with regard to serum levels of VEGF in patients with ovarian tumours.

| Author | Materials | Results |
|---|---|---|
| Boss et al, 2001 | 25 malignant, 12 borderline and 70 benign ovarian tumours | Significant difference between sVEGF of EOC patients versus controls or benign tumours. |
| Chen et al, 1999 | 56 ovarian carcinomas | sVEGF in ovarian cancers higher than in ovarian endometriomas. |
| Cooper et al, 2002 | 34 benign ovarian tumours, 101 invasive EOC | Significant difference between sVEGF of EOC patients versus benign adnexal masses. |
| DeHaven et al, 2002 | Non gynaecological group, benign gynaecological cases and patients with malignancies | No significant difference between the three populations. |
| Demirkiran et al, 2003 | 45 benign cystadenomas and 43 with malignant ovarian carcinomas | Significant difference between sVEGF of EOC patients versus benign cysts. |
| Fasciani et al, 2001 | 9 ovarian carcinomas, 38 endometriomata, 43 serous ovarian cysts and 10 follicular ovarian cysts | Significant difference between sVEGF of EOC patients and endometriomata than in those with benign or follicular cysts. |
| Kraft et al, 1999 | 145 healthy individual, 88 nonmalignant (liver, infectious) disease, 35 ovarian carcinomas | No correlation between age and sVEGF. |
| Obermair et al, 1998 | 131 healthy women, 81 benign ovarian cysts, 44 ovarian | Significant difference between sVEGF of EOC patients versus |

| | | |
|---|---|---|
| | carcinomas | controls. |
| Oehler and Caffier, 1999 | 20 healthy women, 20 benign cystadenomas, 41 EOC | Significant difference between sVEGF of EOC patients versus controls or benign tumours. |
| Yamamoto et al, 1997 | | sVEGF levels were significantly higher in ovarian carcinoma patients than those in patients with borderline or benign tumours. |

**Table 5.19 Studies on sVEGF in patients with ovarian tumours.**

### 5.5.3 sVEGF AND BENIGN CYSTADENOMAS

Studies shown in Table 5.19 showed that sVEGF were significantly higher in EOC patients than those in patients with benign cystadenomas or controls (Yamamoto et al, 1997; Oehler and Caffier, 1999; Fasciani et al, 2001; Boss et al, 2000; Demirkiran et al, 2003). In contrast, Dehaven et al did not find any significant difference in sVEGF levels between normal, benign and malignant ovarian tumours (Dehaven et al, 2002). In this study, sVEGF were also elevated in patients with EOC compared to patients with benign cystadenomas or the control group.

No data concerning VEGF serum levels in patients with different benign histological subtypes have been published yet. In this study, no differences were found in the sVEGF levels between different benign ovarian subtypes, although mucinous subtype showed higher sVEGF than serous subtype.

### 5.5.4 sVEGF AND EOC

In the majority of the studies from Table 5.19, sVEGF levels were found significantly elevated in patients with ovarian carcinoma compared with those with benign cystadenomas and healthy individuals; results which are in agreement with those of this study. These findings suggest the possibility that there may be a greater circulation by ovarian cancers than by benign tumours.

Several authors also found that older patients aged more than 50 years, demonstrate higher sVEGF values than younger patients (Chen et al, 1999; Obermair et al, 1998). My results also demonstrate a significant increase in sVEGF levels in women aged more than 51 years old than in those aged less than 51, however, little is known why older women tend to express higher sVEGF. These results suggest that in older vessels, platelet breakdown is more likely to happen, thus VEGF leaking in blood and hence the increase in sVEGF in women aged more than 51 years.

#### 5.5.4.1 sVEGF AND EOC SUBTYPES

When sVEGF levels were analysed between subtypes, endometrioid and clear cell subtype demonstrated higher sVEGF values compared to other subtypes, although this difference was not significant. From the few studies in the literature, analysing sVEGF with EOC subtypes, they also found no correlation. However, it is worth noting that lower sVEGF levels were reported in serous and mucinous tumours by two different studies (Chen et al, 1999; Obermair et al, 1998), but, neither study specified what they were compared with. This tendency of serous and mucinous subtypes to show low sVEGF values is a reflection of the trend observed in VEGF tissue expression, studied in the previous chapters in which

serous and mucinous subtypes tend to express low levels of VEGF protein. These findings therefore clearly indicate that serous and mucinous EOC subtypes may release less VEGF into the circulation than other subtypes and that the angiogenic activities may be different depending on the histological morphology of the tumours.

5.5.4.2 sVEGF VERSUS STAGE

In this present study, EOC stage I tumours displayed lower sVEGF levels compared to later stages, however this was not significant. This finding is not consistent with other studies demonstrating higher sVEGF levels in early stages of EOC compared with other late stages (Obermair et al, 1998; Cooper et al, 2002). This difference in results may be due to the small numbers of samples analysed in this study, and therefore the question of whether sVEGF levels are indicative of an early or later phenomenon of tumour angiogenesis remains unanswered.

5.5.4.3 sVEGF VERSUS GRADE

From chapter 4, immunohistochemical studies detected VEGF in a higher proportion of grade 2 tumours compared to other grades, however, in this chapter, grade 3 EOC tumours showed higher sVEGF concentrations than the lower grades (1 and 2) of EOC, but this trend did not achieve statistical significance ($p=0.18$). Similar results were observed by several studies (Obermair et al, 1998; Tempfer et al 1998; Chen et al 1999; Cooper et al, 2002) but only two achieved to show a statistically significant correlation between sVEGF levels and grade (Tempfer et al 1998; Chen et al 1999). These differences in the statistical significance of grade versus sVEGF levels may be due to the small numbers of samples analysed and

thus the question of whether sVEGF and protein VEGF levels are indicative of tumour proliferation and differentiation of tumour cells remains unclear.

### 5.5.5 sVEGFR-2

To date, this is the first study reporting sVEGFR-2 levels in healthy women and those with normal ovaries, benign and malignant tumours. This may be explained by the fact that no ELISA kit was available until recently, to measure reliable VEGFR-2 concentrations in serum samples (Ebos et al, 2004). Ebos et al showed that sVEGFR-2 detected in human plasma could now be confirmed using commercially available sVEGFR-2 sandwich ELISA kits designed to detect the extracellular domain of both human and murine VEGFR-2 using monoclonal and polyclonal antibodies (Ebos et al, 2004).

### 5.5.6 sVEGFR-2 AND HEALTHY VOLUNTEERS

Like sVEGF, sVEGFR-2 levels showed similar findings in this group of women, i.e. no statistically significant difference in sVEGFR-2 levels within the length of the cycle but higher sVEGFR-2 levels in early proliferative phase than in secretory phase.

### 5.5.7 sVEGFR-2 AND BENIGN CYSTADENOMAS

In women with benign cystadenomas, sVEGFR-2 showed higher values in pre-menopausal than in post-menopausal women. Again these results are in contradiction with sVEGF which was lower in pre-menopausal than in post-menopausal women. In addition, no correlation was found between sVEGFR-2 and benign cystadenoma subtype. These results may reflect an inverse relationship

between growth factor and receptor, however greater numbers of samples are needed to verify this relationship.

### 5.5.8 sVEGFR-2 AND EOC

In this study, sVEGFR-2 concentrations showed a lower level in EOC compared to controls, although the p value was >0.05. These results are in contradiction with sVEGF results found in this study where a significant increase was reported in EOC compared to controls. Again, more samples are required to study whether there is an inverse relationship between VEGF and VEGFR-2.

Unlike in benign cystadenomas, EOC tumours had higher sVEGFR-2 levels in post-menopausal than in pre-menopausal women, however, this difference was not significant. No correlation was found between sVEGFR-2 and EOC histological parameters such as histological subtype, stage or grade. Due to the non-significance of the p values with regard to sVEGFR-2 in serum, these findings revealed that the evaluation of sVEGFR-2 cannot be used as a potential screening tool for clinical samples in ovarian tumours.

### 5.5.9 OVARIAN FLUIDS

Table 5.20 shows the results of other studies with regard to VEGF levels in ovarian fluids.

| Author | Results |
|---|---|
| Abu-Jawdeh et al, 1996 | VEGF cyst fluids higher in both borderline and malignant tumours than in benign serous cysts. |
| Boss et al, 2001 | Significant difference between VEGF of EOC patients versus controls or benign tumours. |
| Demirkiran et al, 2003 | Significant difference in cyst fluid of VEGF between EOC |

| | |
|---|---|
| | patients and benign cysts. |
| Fasciani et al, 2001 | VEGF higher in cystic fluids of ovarian carcinomas than in serous and follicular cysts. |
| Kraft et al, 1999 | VEGF in malignant effusions 10 fold higher than in matched serum samples |
| Hazelton et al, 1999 | VEGF higher in cystic fluids of ovarian carcinomas than in follicular cysts. |
| Olson et al, 1994 | The malignant epithelium of EOC as one source of VEGF in ascites. |
| Yeo et al, 1993 | Abundant levels of VPF in the ascites of EOC patients. |

**Table 5.20 VEGF levels in ovarian cystic fluids and malignant ovarian effusions.**

A common result from these studies is that VEGF levels are higher in ascitic fluids than in serum samples from EOC patients.

5.5.9.1 CYSTIC FLUIDS

In this study, an average of 4-fold increase of VEGF levels was measured in cystic fluids compared with serum levels, of non-matched patients having benign cystadenomas. Similar observations were described by several authors (Hazelton et al, 1999; Boss et al, 2001; Fasciani et al, 2001; Candido dos Rei, 2002). Work by Fasciani et al, showed an average 6-fold increase in cystic follicular cysts compared with matched serum levels and a 10 fold increase in cystic serous cysts compared with corresponding serum levels. These observations indicate that there is a significant local release of VEGF within the peritoneal cavity. Yamamoto et al believed that this increase in sVEGF production by malignant tumour cells could result in a more pronounced vascular leakage due to VPF action than in the case of non-malignant tumour growth and as a consequence, leakage of VEGF from a highly permeable neo-vascularised tumour results in higher VEGF blood

concentrations (Yamamoto et al, 1997). However, one main drawback of the results in this study was the comparison of cystic fluids with non-matched serum samples as only 4 cystic fluids with matched serum samples were available and this was insufficient for statistical analysis. This may be the reason why the degree of increase between cystic and serum samples is much lower than in other similar studies (Hazelton et al, 1999; Boss et al, 2001; Fasciani et al, 2001; Candido dos Rei, 2002).

5.5.9.2 ASCITIC FLUIDS

In this study, ascitic fluids from EOC patients showed up to 2.3-fold increase compared with non-matched serum samples. Kraft et al found up to 10-fold increase of sVEGF in malignant effusions compared with matched serum samples (Kraft et al, 1999; Harlozinska et al, 2004). Again, the drawback of the findings in this study was the comparison of non-matched samples, showing a lesser increase between ascitic fluids and serum samples than in other studies. However, in all studies including this one, the consistent increase in VEGF levels of several-fold higher in ascitic fluids than in serum samples clearly indicates that ascites may be formed in ovarian malignancies as a result of the neovascularisation of these implants and the increased permeability of these vessels due to the massive local production of VEGF by the tumour itself.

**5.5.10 PLATELET COUNTS AND VEGF**

Banks et al demonstrated that VEGF is contained in platelets and believed that platelets should be taken into account when measuring sVEGF levels (Banks et al, 1998). Following Banks' advice, I compared VEGF levels with platelet counts to

find out any relationship that platelets may have on VEGF. In EOC, both platelet counts and sVEGF values showed the highest values. Like VEGF, platelet count also showed a statistically significant increase from benign to malignant tumours ($p = 0.004$). However when VEGF was correlated with matched platelet counts, no relationship was found ($p>0.05$). This is possibly due to the small number of samples analysed. Therefore, a greater sample number is needed to find out whether platelets may be one of the sources of VEGF measured.

## 5.6 CONCLUDING RESULTS

This has been the first study measuring both VEGF and VEGFR-2 levels in sera, cystic fluid and ascitic fluid of patients with EOC. To a certain extent, soluble VEGF appears to be more important than VEGFR-2 to distinguish between benign and malignant ovarian lesions, and hence to predict malignancy (see Figure 5.18). Albeit the low numbers of tumours available, this study has provided the first observation that clear cell and endometrioid subtypes in EOC showed high VEGF and VEGFR-2 values. The lack of correlation between VEGF and platelet counts in EOC, does not answer the question whether the VEGF levels measured in serum is produced from platelets or from tumour cells. However, although non-matched ascitic fluids and serum samples were used in this study, ascitic fluids in EOC and cystic fluids in benign lesions demonstrated higher VEGF concentrations than serum samples.

In summary, these findings support the assumption that angiogenesis, as reflected by serum VEGF, plays a functional role in ovarian carcinogenesis. However, a possible clinical use of serum VEGF in the diagnosis of EOC progression remains

to be determined. For this reason, serum VEGF should be tested alongside another serum tumour marker such as CA-125.

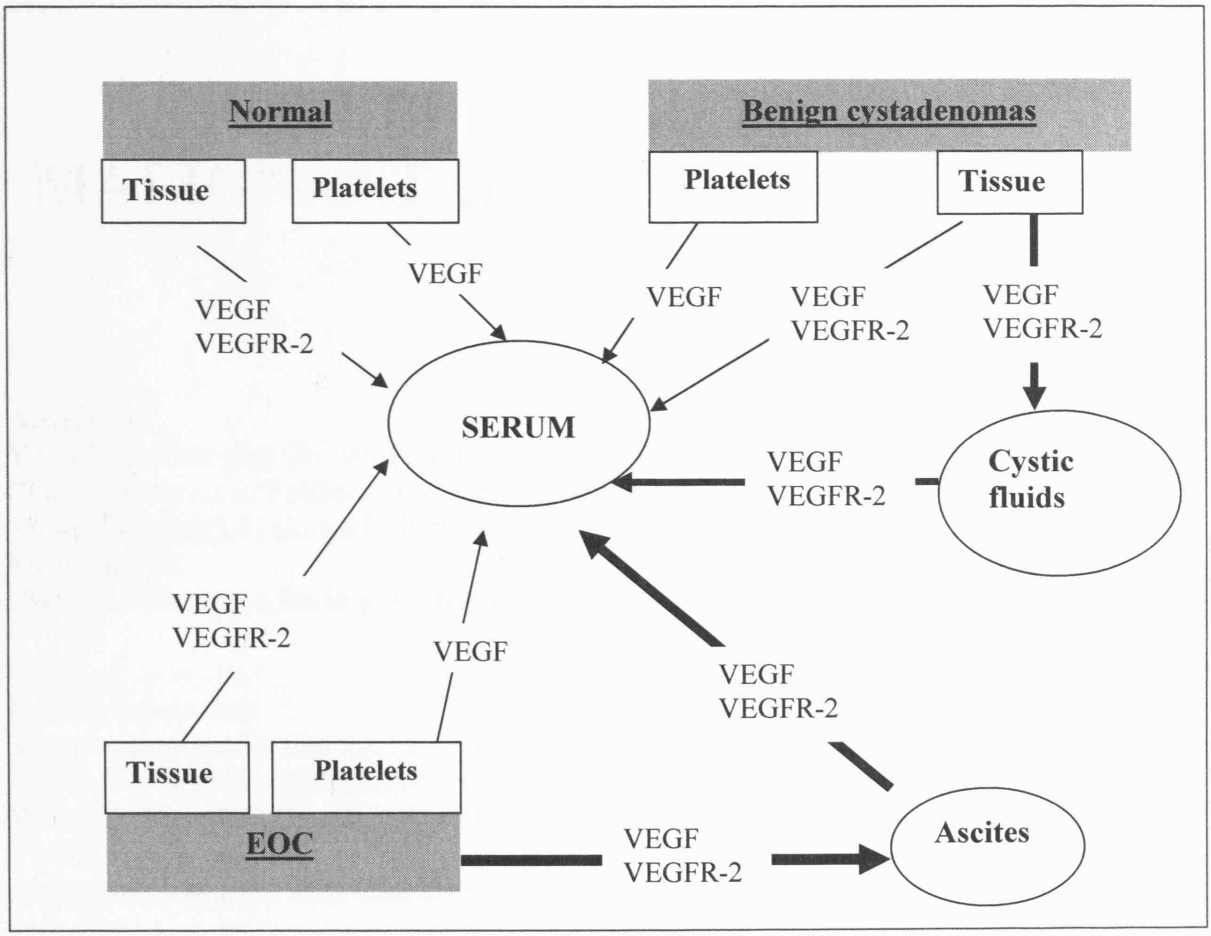



**Figure 5.16 Possible involvement of VEGF and VEGFR-2 levels during EOC progression.**

# CHAPTER 6

# IGF-1 IN BENIGN AND MALIGNANT OVARIAN TISSUES

**Abstracts:**
Vascular Endothelial Growth Factor (VEGF) and Insulin-like Growth Factor-1 (IGF-1) isoforms in Epithelial Ovarian Cancer (EOC).
**Wong Te Fong LF**, Cortes E, Perrett CW, Crow JC, Goldspink G, Yang SY, MacLean AB.
**Blair Bell Research Society Abstracts April 2007.**

**Oral Presentation:**
Vascular Endothelial Growth Factor (VEGF) and Insulin-like Growth Factor-1 (IGF-1) isoforms in Epithelial Ovarian Cancer (EOC).
**Wong Te Fong LF**, Cortes E, Perrett CW, Crow JC, Goldspink G, Yang SY, MacLean AB.
**Proceedings of Blair Bell Research Society Meeting, April 2007, Derby UK.**

## 6.1 INTRODUCTION

The possible involvement of IGF in the development of cancer was initially shown in malignant cell types such as sarcoma, leukaemia and carcinomas of the breast, and colon (Macaulay et al, 1992). To date, the IGF-1 system is strongly implicated in breast and endometrial carcinogenesis as a regulator of the growth of cancer cells (Daughaday, 1990; Cullen et al, 1991; Kleinman et al, 1995; LeRoith et al, 1995; Lee and Yee, 1995).

In general, IGFs interact synergistically with other mitogenic growth factors and steroids and antagonise the effects of anti-proliferative molecules in cancer cells. For example, in breast cancer cells, oestrogens enhance the mitogenic effect of IGF-1 (Stewart et al, 1990; Ruan et al, 1995), increase the levels of IGFBP proteases (Mathieu et al, 1991) and decrease the IGF-2 receptor (Mathieu et al, 1991) and the synthesis of IGFBP (Owens et al, 1993). IGF-1 also enhances the expression of oestrogen receptor in breast cancer cells (Clemmosn et al, 1990). Anti-oestrogenic molecules like tamoxifen abolish the effects of oestrogens on IGF-1 (Huynh et al, 1993; 1996; Pratt et al, 1993), inhibit transcription of IGF-1, and attenuate the response of IGF-IR to IGFs (Huynh et al, 1993; Guvakova et al, 1997).

Recent epidemiological studies have related elevated circulating levels of IGF-1 to an increased risk of cancers of the breast, prostate and colon (Stattin et al, 2000; Toniolo et al, 2000). At present, the principal mechanisms by which IGF-1 is believed to influence cancer risk involves increased cell proliferation, inhibition of apoptosis and stimulation of angiogenesis (Yu and Rohan, 2000). It has been shown that a decrease in serum IGF-1 concentrations reduces its local level and leads to the inhibition of angiogenesis in breast carcinoma (Peyrat et al, 1993).

Furthermore, some studies reported an enhanced expression of VEGF by IGF-1 in colorectal carcinoma cell lines (Warren et al, 1996) and in human and mouse osteoblast-like cells (Goad et al, 1996). Thus in addition to its proliferative functions, IGF-1 appears to partially control angiogenesis, however, little is known about whether there is a link between the underlying mechanisms.

It is now apparent that IGF-1, which is normally located in the liver and is the main regulator of tissue mass during post-natal growth (Stewart and Rotwein, 1996), is also produced by other tissues. Yang et al (1996) reported the non-hepatic expression of IGF-1 splice variants in skeletal muscle after mechanical overload. Hameed et al (2003) confirmed the presence of these splice variants in skelelal muscle after weightlifting exercise. Both studies highlighted that muscle tissue was capable of expressing IGF-1Ec locally in response to mechanical signals and/or damage. The evidence indicates that the splice variants IGF-1Ea and IGF-1Ec have different roles, with the role of IGF-1Ec after muscle injury being to activate muscle satellite cell proliferation. This initial activation of muscle satellite (stem) cells (Hurme et al, 1991; 1992) is important because muscle is a post-mitotic tissue, and the activated satellite cells fuse with the muscle fibres to provide the extra nuclei for muscle fibre repair. The IGF-1 gene is then spliced to IGF-1Ea which recent studies suggest provides the main anabolic response that is involved in generally up-regulating protein synthesis (Hill and Goldspink, 2003). To date, no studies have identified and/or quantified the existence of IGF-1 splice variants in ovarian tissue and possible differential tissue proliferation following this.

## 6.2 AIMS

The aims of this part of the study are to assess the expression of IGF-1 splice variants in response to the transition from normal epithelium to malignant EOC and to investigate its relationship with VEGF and MVD in EOC progression.

## 6.3 MATERIALS AND METHODS

### 6.3.1 ARCHIVAL BLOCKS FOR IHC STUDY

A total of 58 patients, who underwent either hysterectomy or unilateral / bilateral salpingo-oophorectomy, during 1991 to 2001 at the Royal Free Hospital London, were chosen for this study and 18 normal ovarian specimens obtained from women who underwent oophorectomy for non-ovarian conditions (related to leiomyoma, uterine bleeding or pelvic pain) were examined as controls (see Table 6.1). Serial sections of 5 μm from formalin-fixed paraffin wax-embedded tissue samples were prepared. IHC was then performed to detect the protein expression of IGF-1 splice variants (IGF-1 Ea and IGF-1Ec), VEGF and vWF (for MVD analysis) as described in Chapter 2. IHC was repeated three times to ensure consistency and scoring of each marker was as described in Section 2.2.1.5.

| **Diagnosis** | **n** | **Mean age ± SD (range), years** |
|---|---|---|
| **Controls** | 18 | 49.6 ± 11.3 (25-81) |
| **Benign cystadenomas** | 23 | 44.5 ± 12.3 (24-75) |
| **Subtype** | | |
| Serous | 5 | 41.6 ± 12.2 (31-61) |
| Mucinous | 12 | 46.8 ± 14.2 (24-75) |
| Unclassified | 6 | 43.2 ± 9.3 (32-54) |
| **EOC** | 35 | 57.2 ± 14.3 (25-86) |
| **Subtype** | | |
| Serous | 22 | 57.0 ± 13.6 (29-79) |

| | | |
|---|---|---|
| Mucinous | 8 | 52.8 ± 18.1 (25-86) |
| Endometrioid | 4 | 54.8 ± 11.1 (39-64) |
| Clear cell | 1 | 54 |
| **Stage** | | |
| I | 8 | 50.0 ± 15.9 (25-73) |
| II | 1 | 65 |
| III | 24 | 58.7 ± 14.4 (29-86) |
| IV | 2 | 59.0 ± 15.6 (48-70) |
| **Grade** | | |
| 1 | 3 | 54.0 ± 6.3 (49-61) |
| 2 | 12 | 58.9 ± 14.9 (39-79) |
| 3 | 15 | 56.9 ± 12.0 (41-78) |

**Table 6.1 Archival samples of different ovarian tissues used in the study.**

### 6.3.2 FROZEN TISSUES AND RNA EXTRACTION

From the Departmental Tissue Bank, 65 ovarian biopsies were used in this study and they are summarised in Table 6.2.

| **Diagnosis** | **n** |
|---|---|
| Normal ovaries | 20 |
| Benign cystadenomas | 24 |
| EOC | 21 |

**Table 6.2 Frozen ovarian tissues used in the study.**

Total RNA was extracted from these samples with the RNeasy Mini kit (Appendix I) according to the manufacturer's protocol (see Chapter 2 Section 2.2.3.1). The extracted RNA was dissolved in RNase-free water, and the concentration was determined by absorbance at 260 nm, as described in Section 2.2.3.1A.

### 6.3.3 FIRST STRAND cDNA SYNTHESIS

Samples of 0.5μg of total RNA were reverse transcribed into cDNA using Omniscript reverse transcriptase (Appendix I). Details of this procedure were previously described in Chapter 2 Section 2.2.3.3.

### 6.3.4 REAL-TIME QUANTITATIVE RT-PCR

Quantification of the mRNA message encoding the two isoforms of IGF-1 (IGF-1Ea and IGF-1Ec) and VEGF was performed using the LightCycler technology (Roche Diagnostics) with SYBR green I as the method of detection. Details of the method are described in Chapter 2, Section 2.2.3.4. A negative control was present in each run in which the template DNA was replaced with RNase-free water. In addition, to ensure unbiased analysis, real-time quantitative RT-PCR was carried out blind and the identity of the samples was only revealed after the mRNA measurements had been made.

### 6.3.5 STATISTICAL ANALYSES

Associations between IGF-1Ea and IGF-1Ec protein levels and several clinicopathological characteristics, and the expression of IGF-1 splice variants and VEGF were examined using chi-square test and Fisher's exact test as appropriate. Comparison of mean values for MVD between different groups were analysed by the independent *t* test. The mRNA values of these splice variants and VEGF were given in mean ± SD μg X μl RNA. Statistical significance was accepted at the $P<0.05$ level.

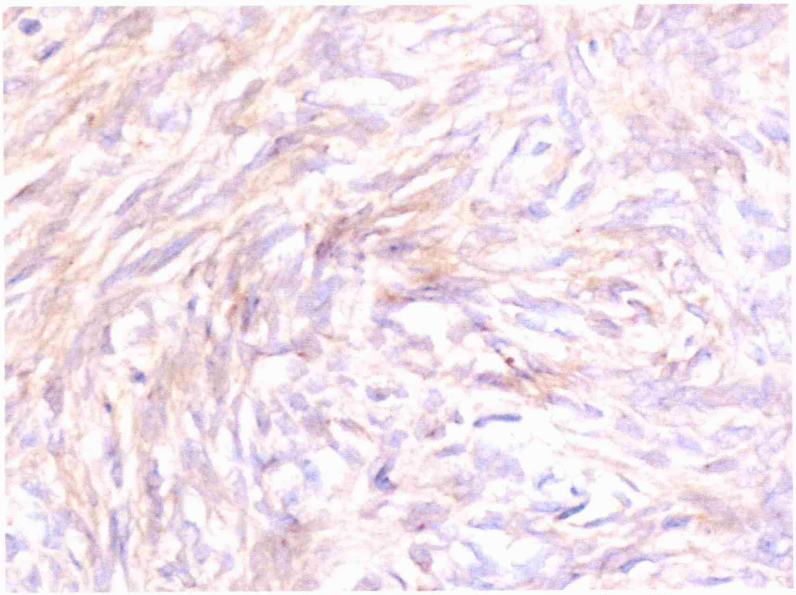
## 6.4 RESULTS

The mean ± SD (range) ages of the different ovarian tissues for IHC study were calculated and are as follows: control group was 49.6 ± 11.3 (25-81), benign group was 44.5 ± 12.3 (24-75), malignant group was 57.2 ± 14.3 (25-86) years (Table 6.1). Because the means of these three age groups had such a wide standard deviation, the differences were not significant.

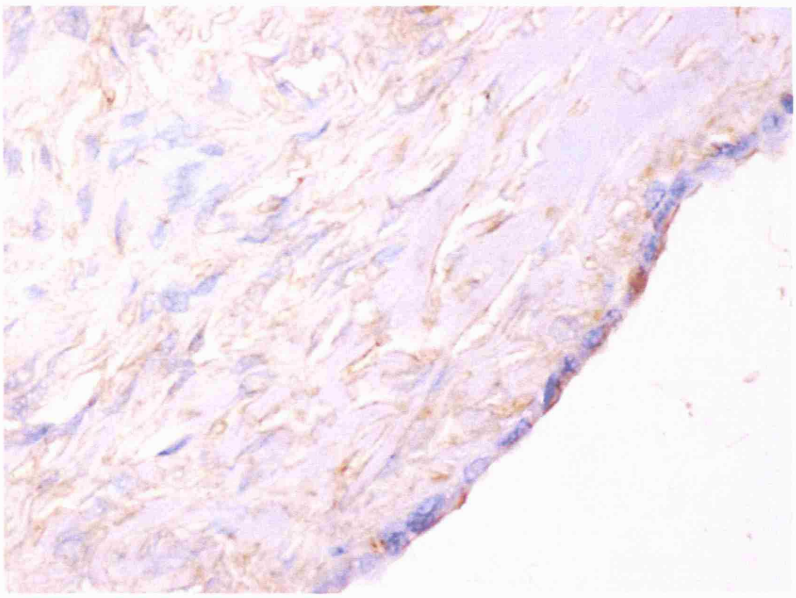
### 6.4.1 IGF-1Ea PROTEIN LEVELS

#### 6.4.1.1 IGF-1Ea PROTEIN LEVELS IN OVARIAN TISSUES

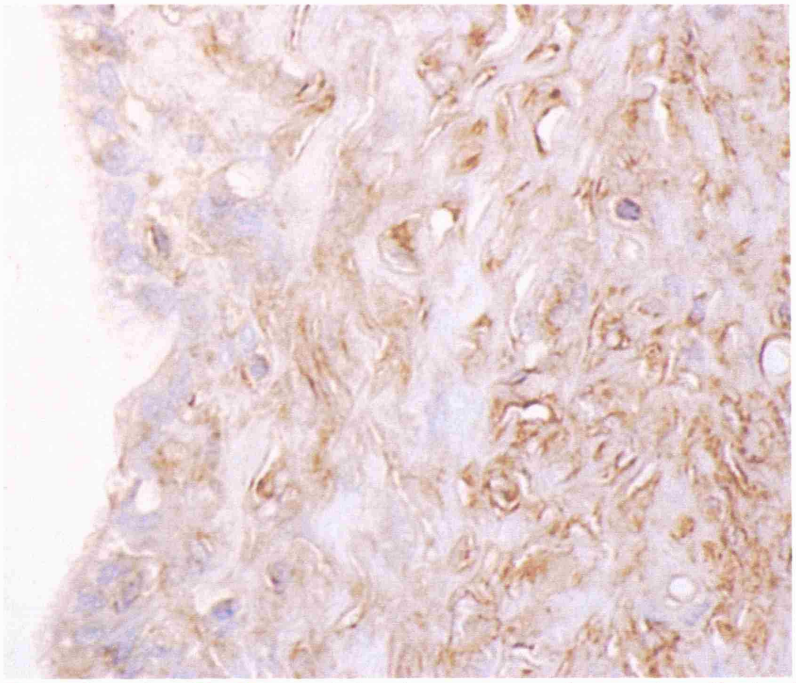
IGF-1Ea expression was found mainly in the cytoplasm of stromal cells, often appearing as a brown wash of staining. Figure 6.1 shows IGF-1Ea staining in different ovarian tissues.



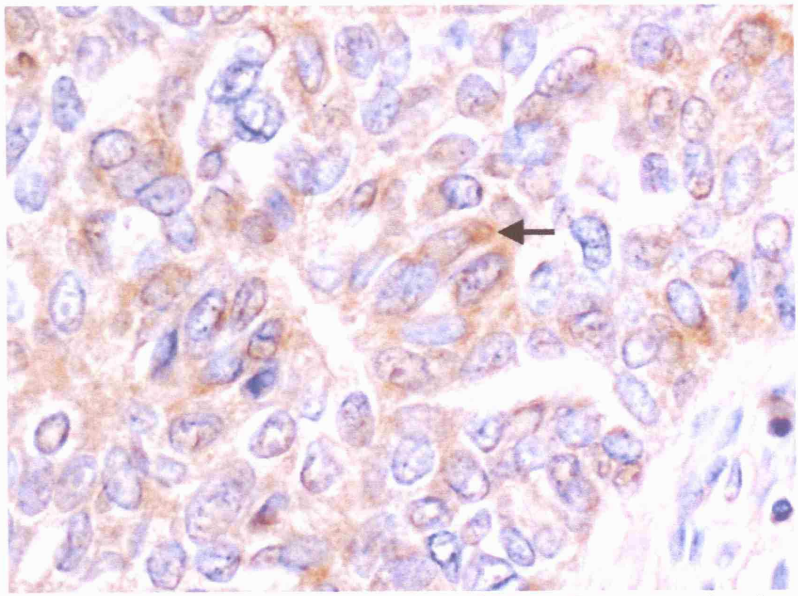
Brown wash staining of IGF-1Ea in the stromal cells.
**Figure 6.1a IGF-1Ea in normal ovary (X400).**



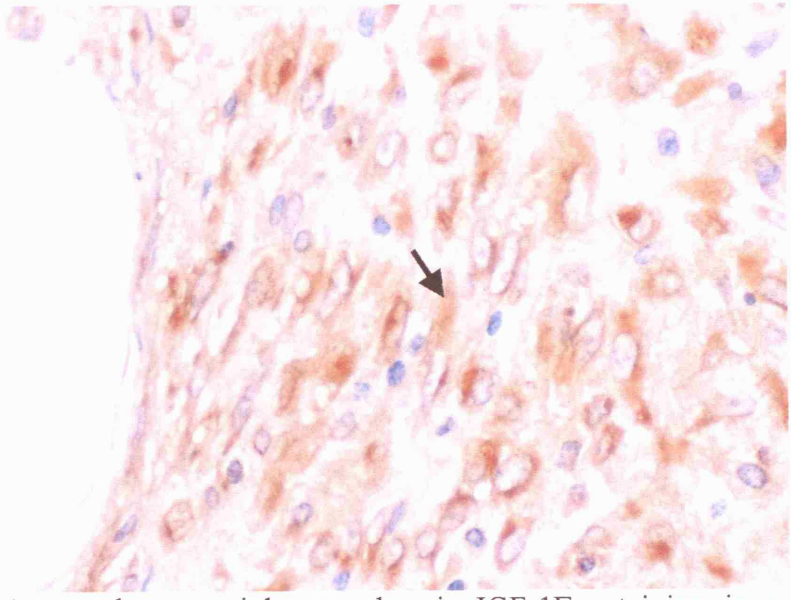
Cytoplasmic IGF-1Ea staining in stroma and occasionally in the epithelium.
**Figure 6.1b IGF-1Ea in serous ovarian cystadenoma (X400).**



Cytoplasmic IGF-1Ea staining in stroma and occasionally in the epithelium.
**Figure 6.1c IGF-1Ea in mucinous ovarian cystadenoma (X400).**



Arrow shows cytoplasmic staining of IGF-1Ea expression in a stromal cell.
**Figure 6.1d IGF-1Ea in serous EOC (X400).**



Arrow shows mainly cytoplasmic IGF-1Ea staining in a stromal cell of the tumour.
**Figure 6.1e IGF-1Ea in mucinous EOC (X400).**

Figure 6.2 shows the percentage of each ovarian tissue type expressing IGF-1Ea. More EOC cases expressed IGF-1Ea compared to controls or benign cystadenomas.

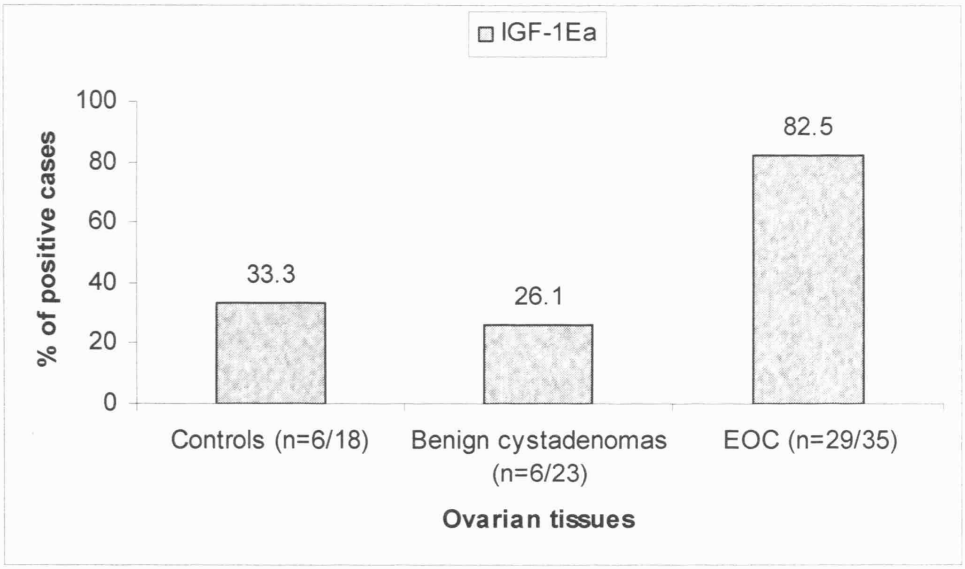



**Figure 6.2 IGF-1Ea expression in different ovarian tissues.**

Table 6.3 shows the statistical comparisons of IGF-1Ea expression between these ovarian tissues and it demonstrates a significant difference in the levels of IGF-1Ea positivity between samples of EOC and benign cystadenomas/normal ovaries.

| **Diagnosis** | **Controls** | **Benign cystadenomas** |
|---|---|---|
| **Benign cystadenomas** | 0.44 | |
| **EOC** | **0.0001** | **0.001** |

**Table 6.3 P values comparing IGF-1Ea expression for each tissue types.**

6.4.1.2 IGF-1Ea PROTEIN LEVELS IN BENIGN CYSTADENOMA SUBTYPES

When benign ovarian cystadenomas were subdivided according to their subtypes, 33.3% of mucinous, but none of the serous, stained positively for IGF-1Ea (see Figure 6.3).

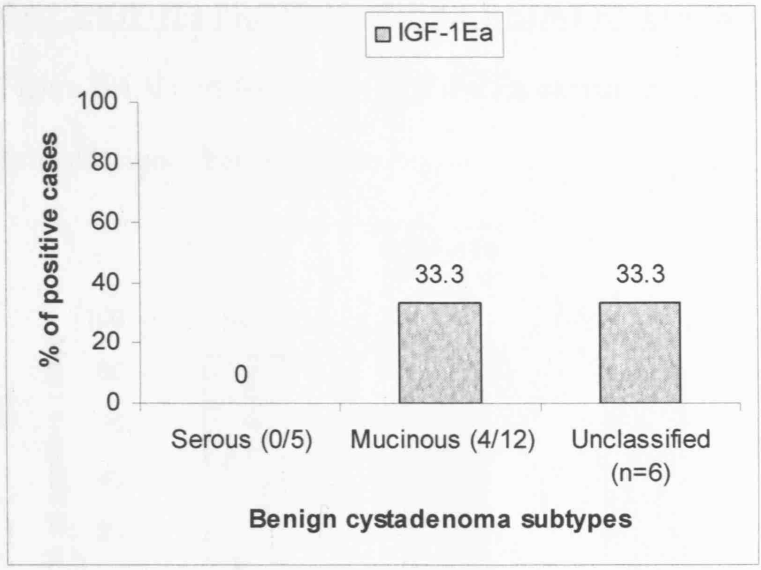



**Figure 6.3 IGF-1Ea expression between benign cystadenoma subtypes.**

Statistical comparisons of IGF-1Ea expression between these benign cystadenoma subtypes show no significant differences of IGF-1Ea expression between these subtypes (Table 6.4). One drawback of this analysis is that the numbers are small once the groups are broken down according to their histological subtypes. This makes the statistical significance difficult to achieve. However, the zero result obtained for serous subtype is interesting and suggests it would be worth increasing the number to see if it still holds true.

| Diagnosis | Serous | Mucinous |
|---|---|---|
| **Mucinous** | 0.33 | |
| **Unclassified** | 0.18 | 0.43 |

**Table 6.4 P values comparing IGF-1Ea expression between benign cystadenoma subtypes.**

6.4.1.3 IGF-1Ea PROTEIN LEVELS BETWEEN EOC SUBTYPES

Figure 6.4 shows the results of IGF-1Ea expression between EOC subtypes with little difference between them.

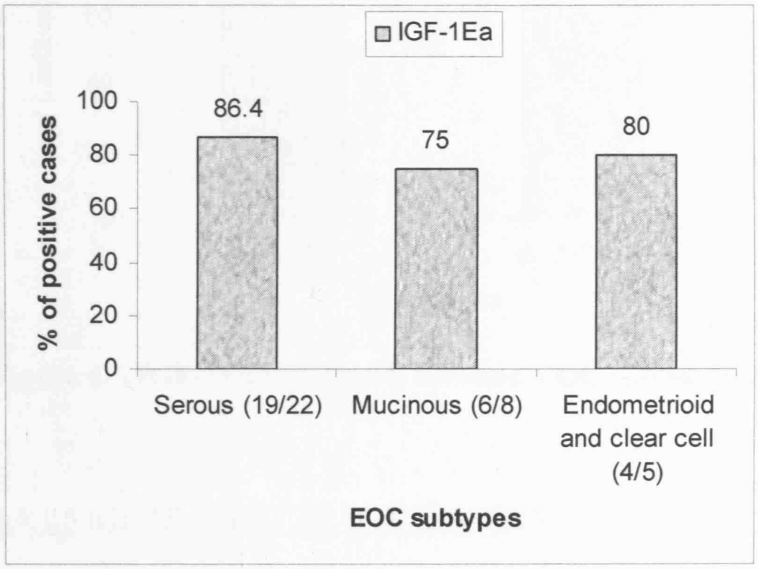



**Figure 6.4 IGF-1Ea expression between EOC subtypes.**

The results of the statistical analysis of IGF-1Ea between EOC subtypes are given in Table 6.5, showing no significant differences in IGF-1Ea expression between these subtypes.

| **Diagnosis** | **Serous** | **Mucinous** |
|---|---|---|
| **Mucinous** | 0.41 | |
| **Endometrioid and clear cell** | 0.58 | 0.69 |

**Table 6.5 P values comparing IGF-1Ea expression between EOC subtypes.**

6.4.1.4 IGF-1Ea PROTEIN LEVELS BETWEEN EOC STAGES

Figure 6.5 shows that 77.8 % tumours of early stages (I and II) and 84.6 % of late stages (III-IV) expressed IGF-1Ea. This difference in percentage was not statistically significant as the p value was 0.49, using Fisher's exact test.

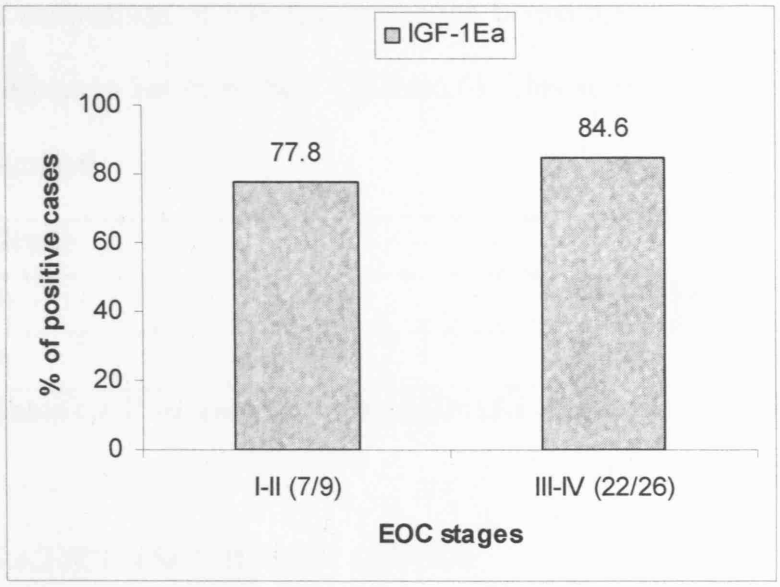



**Figure 6.5 IGF-1Ea expression between EOC tumour stages.**

6.4.1.5 IGF-1Ea PROTEIN LEVELS BETWEEN EOC GRADES

When EOC samples were subdivided according to disease grade, IGF-1Ea expression was expressed in all three grades (Figure 6.6).

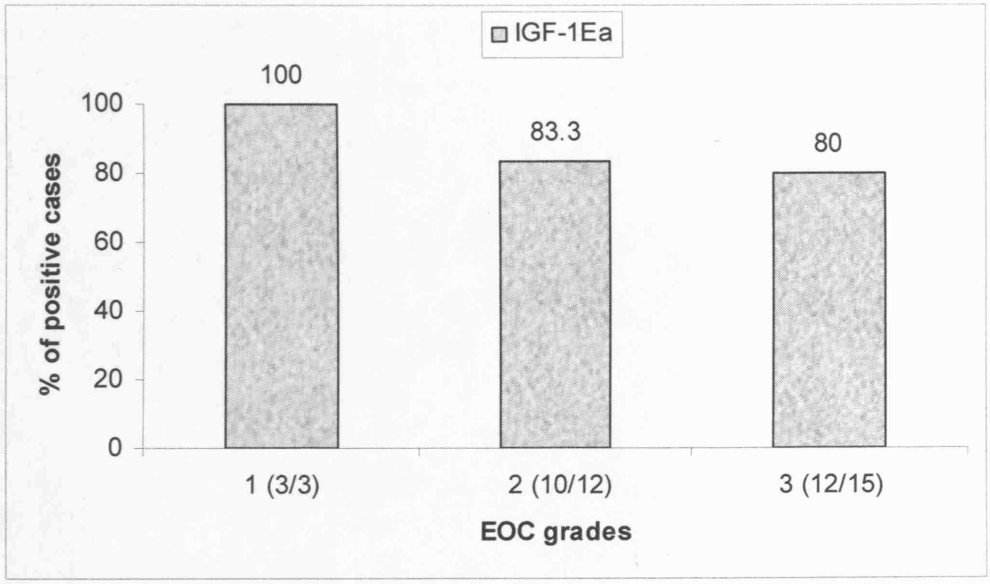



**Figure 6.6 IGF-1Ea expression between EOC tumour grades.**

A comparison of IGF-IEa expression between EOC grades shows no significant difference between them (Table 6.6). This is possibly due to small number of samples.

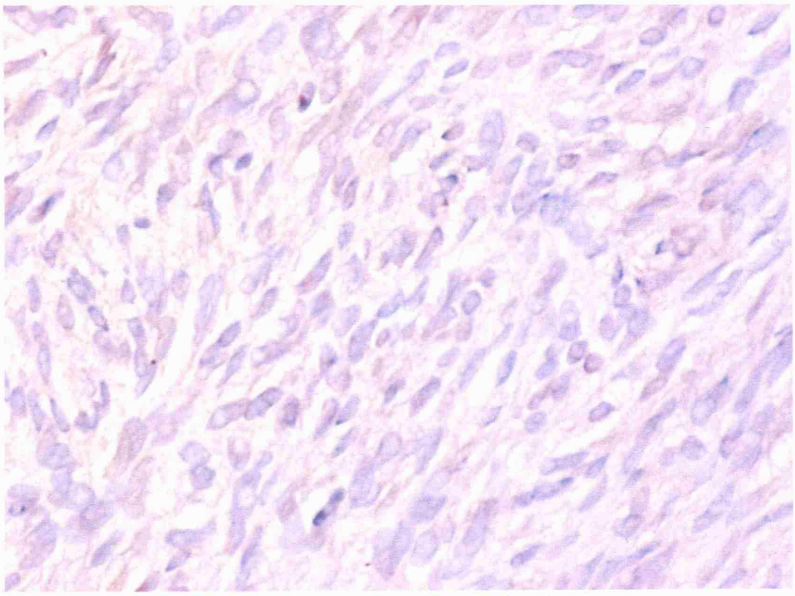
| Grade | 1 | 2 |
|---|---|---|
| 2 | 0.63 | |
| 3 | 0.56 | 0.61 |

**Table 6.6 P values comparing IGF-1Ea expression between EOC grades.**

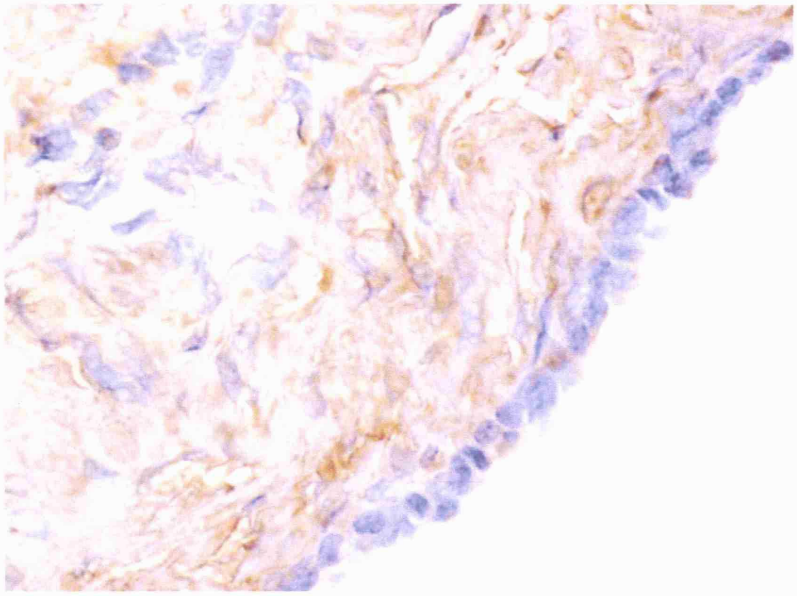
### 6.4.2 IGF-1Ec PROTEIN LEVELS

#### 6.4.2.1 IGF-1Ec PROTEIN LEVELS IN OVARIAN TISSUES

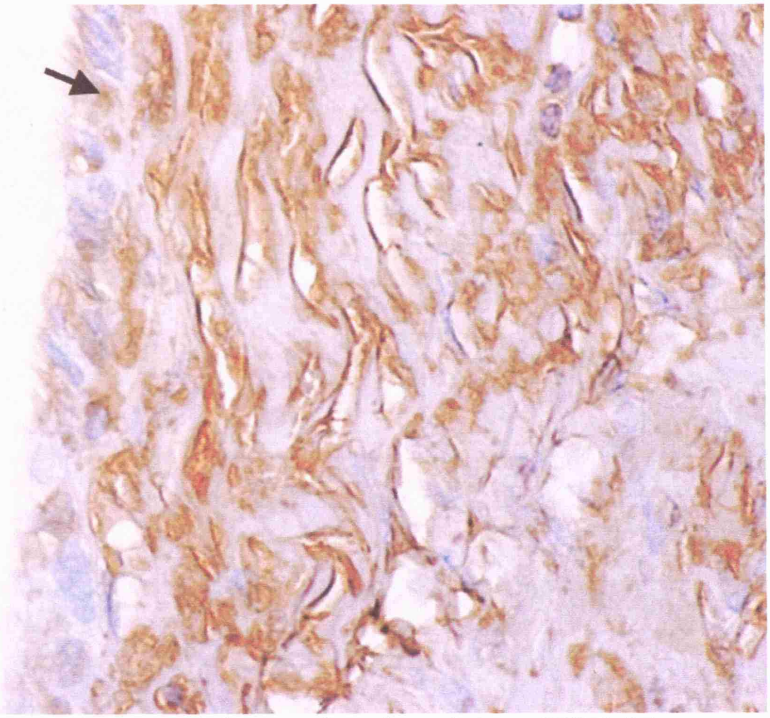
Like IGF-1Ea, IGF-1Ec expression was observed mainly in the cytoplasm of stromal cells. Figure 6.7 shows IGF-1Ec immunostaining in different ovarian tissues.



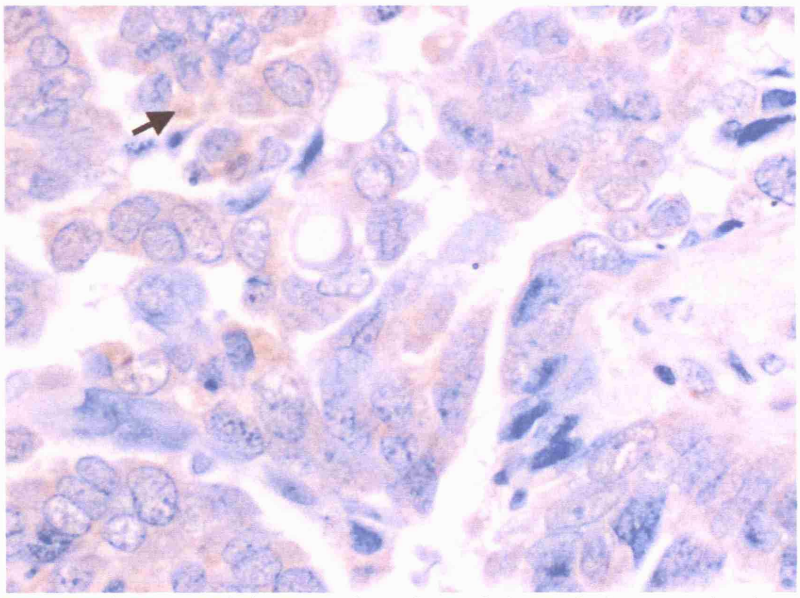
Faint brown wash staining of IGF-1Ec in the stroma.
**Figure 6.7a IGF-1Ec in normal ovary (X400).**



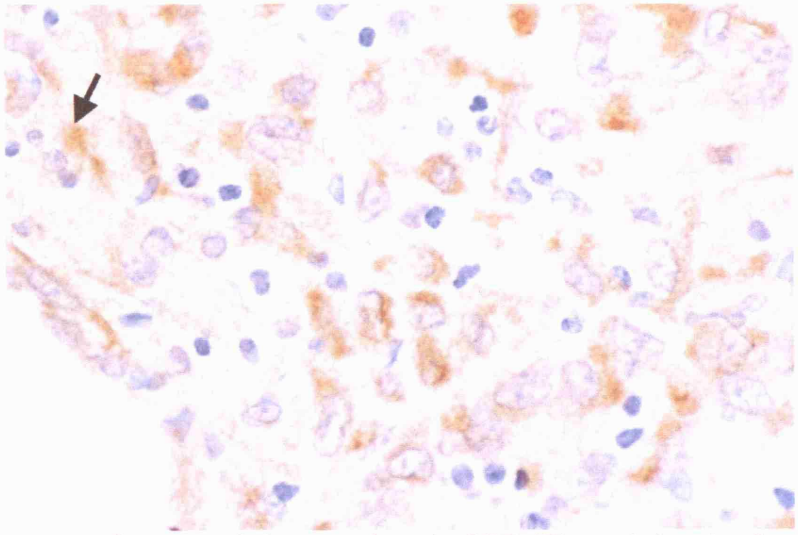
Cytoplasmic staining of IGF-1Ec in the stroma of the tumour.
**Figure 6.7b IGF-1Ec in serous ovarian cystadenoma (X400).**



Arrow shows mainly cytoplasmic staining of IGF-1Ec.
**Figure 6.7c IGF-1Ec in mucinous ovarian cystadenoma, present in both tumour and stromal cells (X400).**



Arrow shows mainly cytoplasmic staining of IGF-1Ec in the stroma.
**Figure 6.7d IGF-1Ec in serous EOC (X400).**



Arrow shows mainly cytoplasmic IGF-1Ec staining in the stromal cells of the tumour.
**Figure 6.7e IGF-1Ec in mucinous EOC (X400).**

Figure 6.8 shows the percentage of each ovarian tissue expressing IGF-1Ec. More benign cystadenoma cases expressed IGF-IEc compared to controls or EOC.

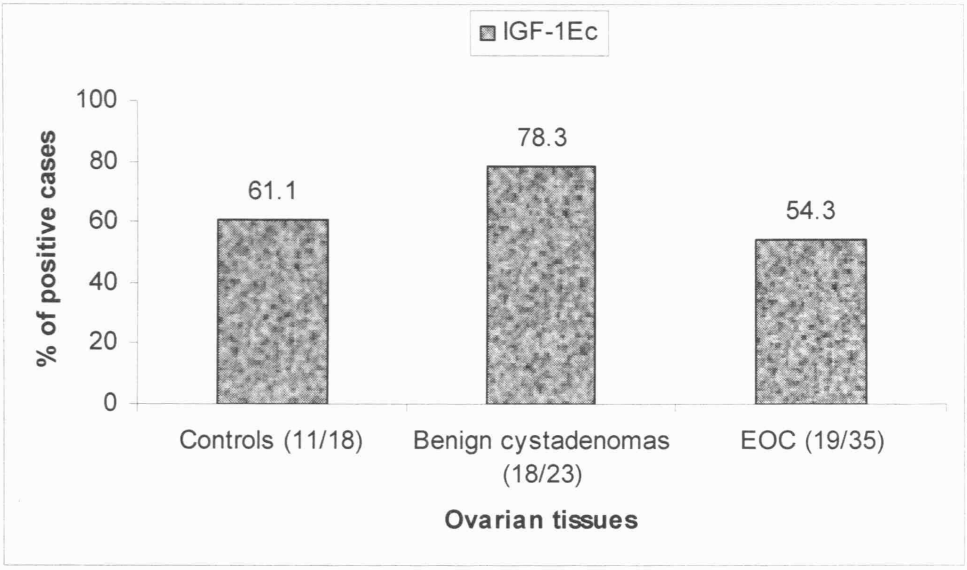



**Figure 6.8 IGF-1Ec expression in different ovarian tissues.**

Statistical comparisons of IGF-1Ec expression between these ovarian tissues showed no significant difference in IGF-1Ec expression between these tissues (Table 6.7).

| Diagnosis | Controls | Benign cystadenomas |
|---|---|---|
| **Benign cystadenomas** | 0.20 | |
| **EOC** | 0.49 | 0.07 |

**Table 6.7 P values comparing IGF-1Ec expression for each tissue type.**

6.4.2.2 IGF-1Ec PROTEIN LEVELS IN BENIGN CYSTADENOMA SUBTYPES

When benign ovarian cystadenomas were subdivided according to their subtypes, more mucinous cystadenomas stained positively for IGF-1Ec compared to serous cystadenomas (see Figure 6.9). This difference was not statistically significant (Table 6.8).

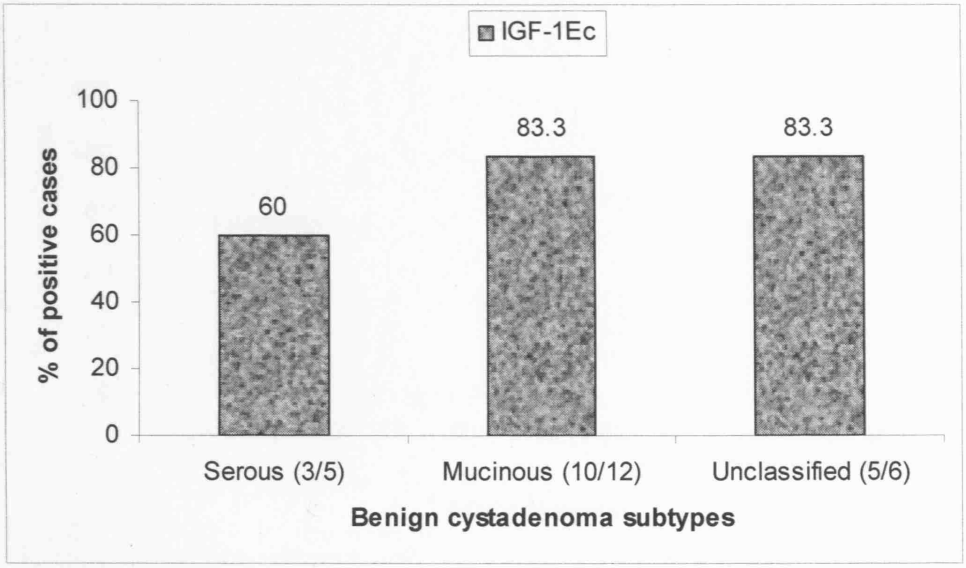



**Figure 6.9 IGF-1Ec expression between benign cystadenoma subtypes.**

| Diagnosis | Serous | Mucinous |
|---|---|---|
| Mucinous | 0.33 | |
| Unclassified | 0.42 | 0.73 |

**Table 6.8 P values comparing IGF-1Ec expression between benign cystadenoma subtypes.**

6.4.2.3 IGF-1Ec PROTEIN LEVELS BETWEEN EOC SUBTYPES

When EOC were subdivided according to their subtypes, less mucinous cases stained positively for IGF-1Ec compared to serous, endometrioid and clear cell carcinomas, as shown in Figure 6.10.

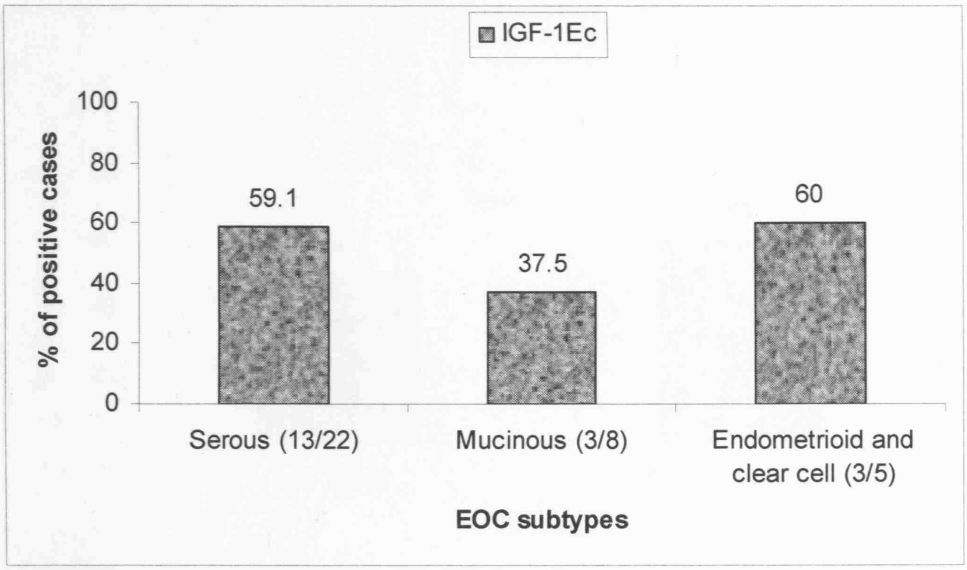



**Figure 6.10 IGF-1Ec expression between EOC subtypes.**

Statistical analysis of IGF-1Ec expression between these EOC subtypes showed no correlation in IGF-1Ec expression between these subtypes (Table 6.9).

| **Diagnosis** | **Serous** | **Mucinous** |
|---|---|---|
| **Mucinous** | 0.26 | |
| **Endometrioid and clear cell** | 0.68 | 0.41 |

**Table 6.9 P values comparing IGF-1Ec expression between EOC subtypes.**

6.4.2.4 IGF-1Ec PROTEIN LEVELS BETWEEN EOC STAGES

Figure 6.11 shows no significant difference in IGF-1Ec expression between EOC stages I-II and stages III-IV, as the p value was 0.46.

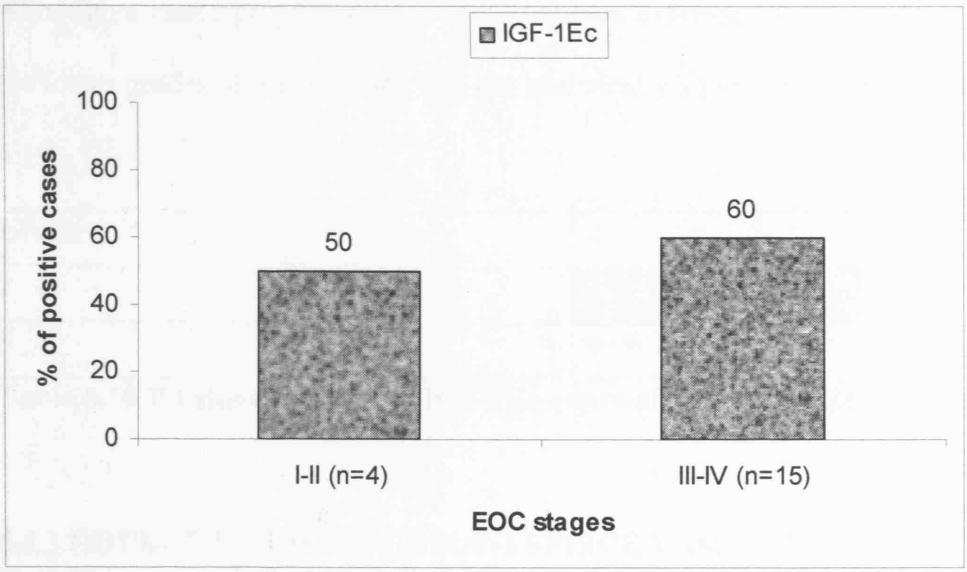



**Figure 6.11 IGF-1Ec expression between EOC tumour stages.**

6.4.2.5 IGF-1Ec PROTEIN LEVELS BETWEEN EOC GRADES

When EOC samples were subdivided between histological grades, IGF-1Ec was positive in 83.3% of grade 2 tumours compared to other grades (Figure 6.12).

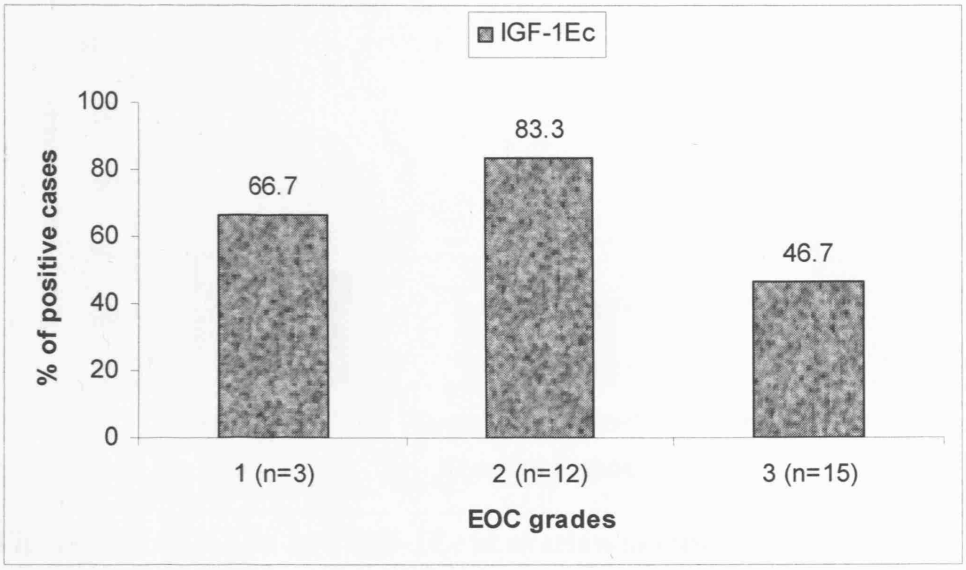



**Figure 6.12 IGF-1Ec expression between EOC tumour grades.**

Although a lesser proportion of grade 3 tumours expressed IGF-1Ec compared to the lower grades, this difference was not statistically significant as shown in Table 6.10.

| Grade | 1 | 2 |
|---|---|---|
| 2 | 0.52 | |
| 3 | 0.50 | 0.06 |

**Table 6.10 P values comparing IGF-1Ec expression between EOC grades.**

### 6.4.3 DOUBLE MARKERS OF IGF-1 SPLICE VARIANTS

#### 6.4.3.1 IGF-1Ea AND IGF-1Ec IN OVARIAN TISSUES

An analysis of the expression of IGF-1Ea and IGF-1Ec together in ovarian tissues showed that more EOC cases were positive for both IGF-1Ea and IGF-1Ec expression compared to controls or benign cystadenomas (Figure 6.13).

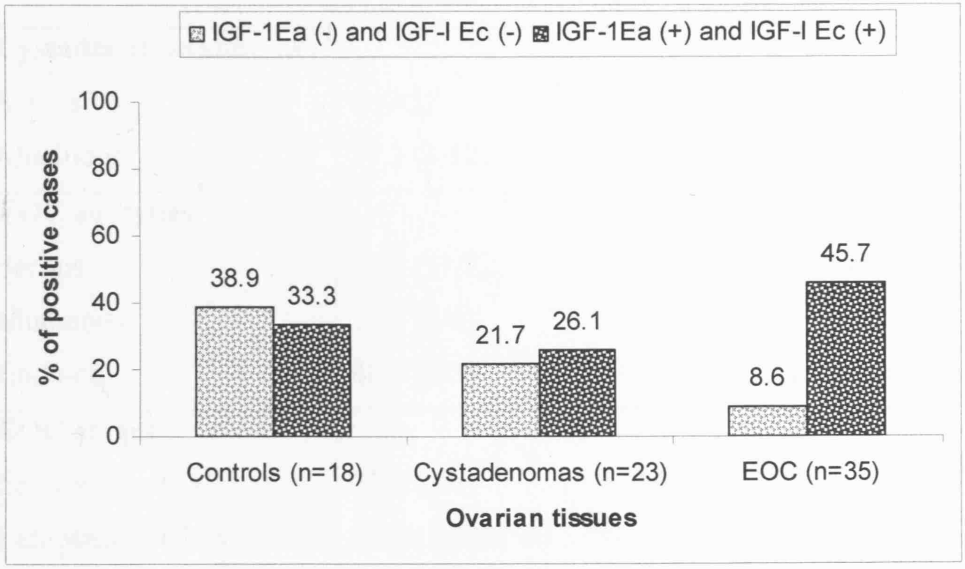



**Figure 6.13 IGF-1Ea and IGF-1Ec in ovarian tissues.**

When statistical analysis was performed between the expression of these double markers and ovarian tissues, a significant difference was found between EOC and

the control group with respect to both IGF-1 splice variants expression (Table 6.11).

| Diagnosis | Controls | Cystadenomas |
|---|---|---|
| **Cystadenomas** | 0.26 | |
| **EOC** | **0.04** | 0.22 |

**Table 6.11 Correlations between IGF-1 splice variants and ovarian tissues.**

6.4.3.2 IGF-1Ea AND IGF-1Ec BETWEEN HISTOPATHOLOGICAL FEATURES

Table 6.12 shows the expression of both IGF-1Ea and IGF-1Ec between benign cystadenomas and EOC histological features, in which no correlation was found for both IGF-1 splice variants expressions between subtypes, stages or grades.

| | **IGF-1Ea (+) and IGF-1Ec (+)** % (fraction) of positive cases | **P value (Fisher's test)** |
|---|---|---|
| **Cystadenomas subtypes** | | |
| Serous | 0 (0/5) | |
| Mucinous | 33.3 (4/12) | 0.28 (serous vs mucinous) |
| **EOC subtypes** | | |
| Serous | 50.0 (11/22) | 0.26 (serous vs mucinous) |
| Mucinous | 37.5 (3/8) | 0.45 (mucinous vs endo-cc) |
| Endo-cc | 40.0 (2/5) | 0.78 (serous vs endo-cc) |
| **EOC stages** | | |
| Early stages (I-II) | 37.5 (3/8) | |
| Late stages (III-IV) | 52.0 (13/25) | 0.76 (early vs late) |
| **EOC grades** | | |
| 1 | 66.7 (2/3) | 0.76 (1 vs 2) |
| 2 | 66.7 (8/12) | 0.25 (2 vs 3) |
| 3 | 40.0 (6/15) | 0.64 (1 vs 3) |

**Table 6.12 Correlations between IGF-1 splice variants and histological features.**

### 6.4.4 DOUBLE MARKERS OF IGF-1Ea AND VEGF

#### 6.4.4.1 IGF-1Ea AND VEGF IN OVARIAN TISSUES

Figure 6.14 showed the immuno-positivity of both IGF-1Ea and VEGF in the ovarian tissues, with a higher proportion of EOC expressing both markers compared to cystadenomas and controls.

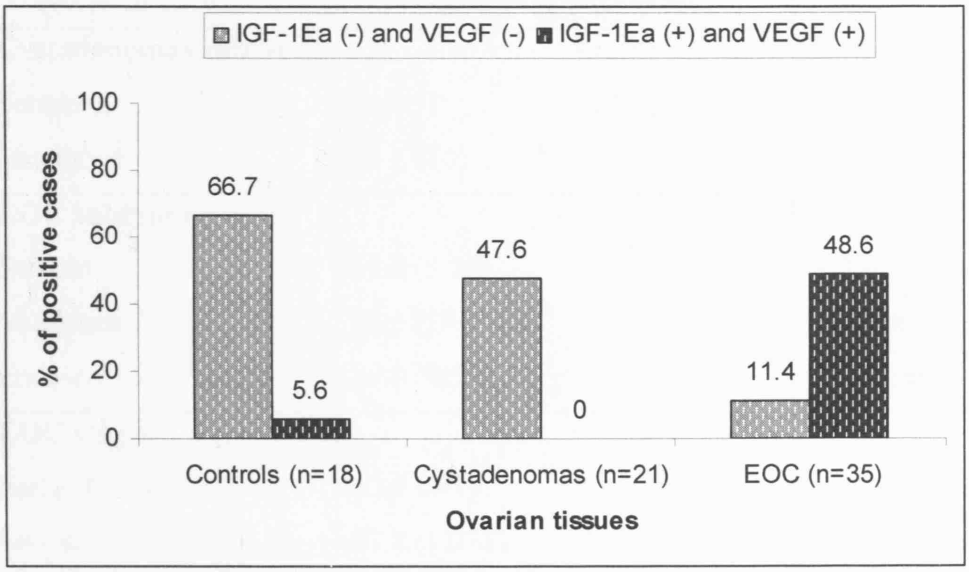



**Figure 6.14 IGF-1Ea and VEGF immunopositivity in ovarian tissues.**

Table 6.13 shows the statistical comparisons of both IGF-1Ea and VEGF expressions between the ovarian tissues, revealing significant differences of both markers between EOC and cystadenomas / controls.

| Diagnosis | Controls | Cystadenomas |
|---|---|---|
| **Cystadenomas** | 0.07 | |
| **EOC** | **0.0001** | **0.0001** |

**Table 6.13 Comparisons of double markers IGF-1Ea and VEGF between ovarian tissues.**

6.4.4.2 IGF-1Ea AND VEGF BETWEEN HISTOPATHOLOGICAL FEATURES

Table 6.14 demonstrates the immunopositivity of IGF-1Ea and VEGF according to benign cystadenomas and EOC histological features, in which no correlation was found for both markers between subtypes, stages or grades.

| | **IGF-1Ea (+) and VEGF (+)** % (fraction) of positive cases | **P value** |
|---|---|---|
| **Cystadenomas subtypes** | | |
| Serous | 0 (0/5) | |
| Mucinous | 30 (3/10) | 0.39 (serous vs mucinous) |
| **EOC subtypes** | | |
| Serous | 50.0 (9/18) | 0.27 (serous vs mucinous) |
| Mucinous | 25.0 (2/8) | 0.06 (mucinous vs endo-cc) |
| Endo-cc | 80.0 (4/5) | 0.42 (serous vs endo-cc) |
| **EOC stages** | | |
| Early stages (I-II) | 62.5 (5/8) | |
| Late stages (III-IV) | 47.8 (11/23) | 0.70 (early vs late) |
| **EOC grades** | | |
| 1 | 33.3 (1/3) | 0.67 (1 vs 2) |
| 2 | 50.0 (5/10) | 0.84 (2 vs 3) |
| 3 | 61.5 (8/13) | 0.31 (1 vs 3) |

**Table 6.14 Comparisons of IGF-1Ec and VEGF between histological features.**

## 6.4.5 DOUBLE MARKERS OF IGF-1Ec AND VEGF

6.4.5.1 IGF-1Ec AND VEGF IN OVARIAN TISSUES

When IGF-1Ec and VEGF were analysed in the ovarian tissues, the results showed more EOC cases positive for both IGF-1Ec and VEGF (Figure 6.15).

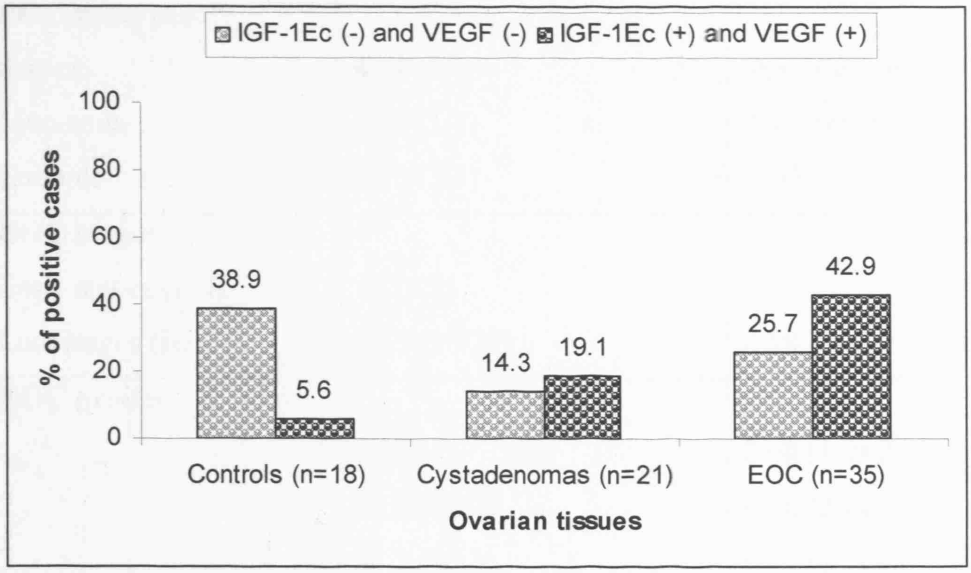



**Figure 6.15 IGF-1Ec and VEGF immunopositivity in ovarian tissues.**

Statistical comparisons of IGF-1Ec and VEGF expressions between these ovarian tissues, revealed a significant difference with both markers between EOC and cystadenomas / controls (Figure 6.15).

| **Diagnosis** | **Controls** | **Cystadenomas** |
|---|---|---|
| **Cystadenomas** | 0.15 | |
| **EOC** | **0.001** | **0.009** |

**Table 6.15 Comparisons of IGF-1Ec and VEGF between ovarian tissues.**

6.4.5.2 IGF-1Ec AND VEGF AND HISTOPATHOLOGICAL FEATURES

The expressions of IGF-1Ec and VEGF according to benign cystadenomas and EOC histological features are shown in Table 6.16, in which no differences were found for both markers between subtypes, stages or grades.

| | **IGF-1Ec (+) and VEGF (+)** % (fraction) of positive cases | **P value** |
|---|---|---|
| **Cystadenomas subtypes** | | |
| Serous | 20 (1/5) | |
| Mucinous | 20 (2/10) | 0.86 (serous vs mucinous) |

| | | |
|---|---|---|
| **EOC subtypes** | | |
| Serous | 44.4 (8/18) | 0.35 (serous vs mucinous) |
| Mucinous | 12.5 (1/8) | 0.07 (mucinous vs endo-cc) |
| Endo-cc | 75.0 (3/4) | 0.37 (serous vs endo-cc) |
| **EOC stages** | | |
| Early stages (I-II) | 50 (4/8) | |
| Late stages (III-IV) | 43.5 (10/23) | 0.58 (early vs late) |
| **EOC grades** | | |
| 1 | 0 (0/3) | 0.06 (1 vs 2) |
| 2 | 60.0 (6/10) | 0.26 (2 vs 3) |
| 3 | 46.2 (6/13) | 0.09 (1 vs 3) |

**Table 6.16 Comparisons of IGF-1Ec and VEGF between histological features.**

### 6.4.6 IGF-1Ea, IGF-1Ec AND VEGF

When a correlation of all three markers was done together, more EOC cases were positive for all three proteins compared with controls or cystadenomas. This was statistically significant (p=0.02). Interestingly, none of the 21 cystadenomas were positive for all three markers.

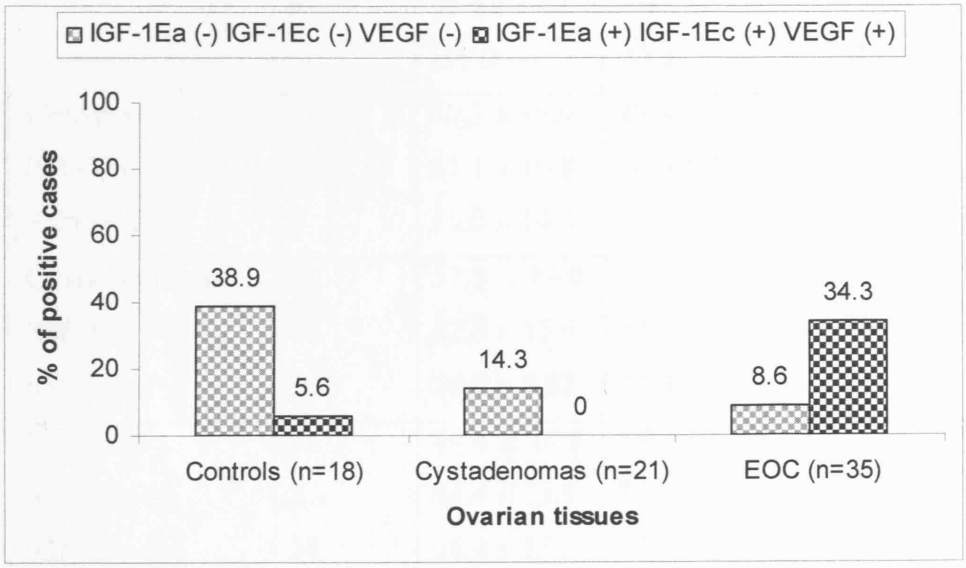



**Figure 6.16 IGF-1Ea, IGF-1Ec and VEGF in ovarian tissues.**

### 6.4.7 IGF-1 splice variants and MVD

In order to understand the contribution of IGF-1 alone or together with VEGF to the stimulation of angiogenesis which might contribute to tumour growth in EOC, this section has analysed MVD in each category of ovarian tissues expressing IGF-1 alone or IGF-1 and VEGF together.

#### 6.4.7.1 IGF-1Ea AND MVD IN OVARIAN TISSUES

Table 6.17 shows the values of MVD in positive and negative cases for IGF-1Ea of the ovarian tissues analysed. No significant difference was found between positive and negative cases for each group, although there is a trend for positive cases to have lower MVD than negative cases for IGF-1Ea. In addition, EOC showed lower MVD values than controls or cystadenonomas, but this was not significant too (p=0.53 and 0.55 respectively). Comparisons of IGF-1Ea and MVD between histopathological parameters did not reach significance (data not shown).

| | n | Mean ± SD values of | | P value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| **Controls** | 17 | 59.3 ± 15.9 | 48.4 ± 12.9 | | |
| IGF-1Ea (-) | 12 | 61.1 ± 16.8 | 50.5 ± 12.9 | 0.49 | 0.44 |
| IGF-1Ea (+) | 5 | 55.0 ± 14.5 | 44.9 ± 13.5 | | |
| **Cystadenomas** | 21 | 51.5 ± 14.0 | 43.4 ± 11.6 | | |
| IGF-1Ea (-) | 16 | 52.0 ± 15.6 | 44.8 ± 12.9 | 0.79 | 0.32 |
| IGF-1Ea (+) | 5 | 50.0 ± 7.52 | 38.8 ± 2.63 | | |
| **EOC** | 35 | 44.4 ± 14.8 | 37.3 ± 12.4 | | |
| IGF-1Ea (-) | 7 | 44.4 ± 13.5 | 37.4 ± 11.9 | | |
| IGF-1Ea (+) | 28 | 44.4 ± 15.3 | 37.2 ± 12.8 | 0.95 | 0.98 |

**Table 6.17 IGF-1Ea and MVD in ovarian tissues.**

6.4.7.2 IGF-1Ec AND MVD IN OVARIAN TISSUES

Table 6.18 shows no significant difference between positive and negative cases for IGF-1Ec within each group analysed. Again, comparisons of IGF-1Ec and MVD between histopathological features did not show any correlation (data not shown).

| | n | Mean ± SD values of | | P value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| **Controls** | 17 | | | | |
| IGF-1Ec (-) | 7 | 57.6 ± 15.0 | 47.3 ± 9.60 | | |
| IGF-1Ec (+) | 10 | 60.5 ± 17.3 | 49.9 ± 15.2 | 0.72 | 0.69 |
| **Cystadenomas** | 21 | | | | |
| IGF-1Ec (-) | 5 | 54.0 ± 15.5 | 47.2 ± 13.9 | | |
| IGF-1Ec (+) | 16 | 50.8 ± 13.9 | 42.2 ± 11.0 | 0.66 | 0.42 |
| **EOC** | 35 | | | | |
| IGF-1Ec (-) | 16 | 46.6 ± 15.5 | 38.6 ± 13.7 | | |
| IGF-1Ec (+) | 19 | 42.6 ± 14.3 | 36.2 ± 11.5 | 0.44 | 0.59 |

**Table 6.18 IGF-1Ec and MVD in ovarian tissues.**

6.4.7.3 IGF-1Ea, IGF-1Ec AND MVD IN OVARIAN TISSUES

When MVD were compared between positive and negative cases for IGF-1Ea and IGF-1Ec, no correlation was found, as demonstrated in Table 6.19.

| | n | Mean ± SD values of | | P value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| **Controls** | 17 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) | 7 | 57.6 ± 15.0 | 47.3 ± 9.60 | | |
| IGF-1Ea (+) IGF-1Ec (+) | 5 | 55.0 ± 14.5 | 44.9 ± 13.5 | 0.77 | 0.73 |
| **Cystadenomas** | 21 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) | 5 | 54.0 ± 15.5 | 47.2 ± 13.9 | | |
| IGF-1Ea (+) IGF-1Ec (+) | 5 | 50.0 ± 7.5 | 38.8 ± 2.6 | 0.62 | 0.22 |
| **EOC** | 35 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) | 4 | 51.3 ± 14.8 | 42.4 ± 14.3 | | |
| IGF-1Ea (+) IGF-1Ec (+) | 16 | 44.0 ± 15.3 | 37.3 ± 12.3 | 0.40 | 0.48 |

**Table 6.19 IGF-1Ea and IGF-1Ec compared with MVD in ovarian tissues.**

6.4.7.4 IGF-1 SPLICE VARIANTS, VEGF AND MVD IN OVARIAN TISSUES

A comparison of MVD values between positive and negative cases for IGF-1Ea, IGF-1Ec and VEGF showed no correlation in MVD between these three markers (Table 6.20).

| | n | Mean ± SD values of | | P value | |
|---|---|---|---|---|---|
| | | HVD | AVD | HVD | AVD |
| **Controls** | 17 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) VEGF (-) | 5 | 66.0 ± 19.7 | 47.3 ± 9.60 | | |
| IGF-1Ea (+) IGF-1Ec (+) VEGF (+) | 1 | 80.0 | 69.0 | 0.55 | 0.48 |
| **Cystadenomas** | 21 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) VEGF (-) | 7 | 54.0 ± 15.5 | 47.2 ± 13.9 | | |
| IGF-1Ea (+) IGF-1Ec (+) VEGF (+) | 0 | - | - | - | - |
| **EOC** | 35 | | | | |
| IGF-1Ea (-) IGF-1Ec (-) VEGF (-) | 7 | 40.1 ± 12.5 | 33.2 ± 10.0 | | |
| IGF-1Ea (+) IGF-1Ec (+) VEGF (+) | 11 | 47.8 ± 14.3 | 39.9 ± 10.9 | 0.26 | 0.21 |

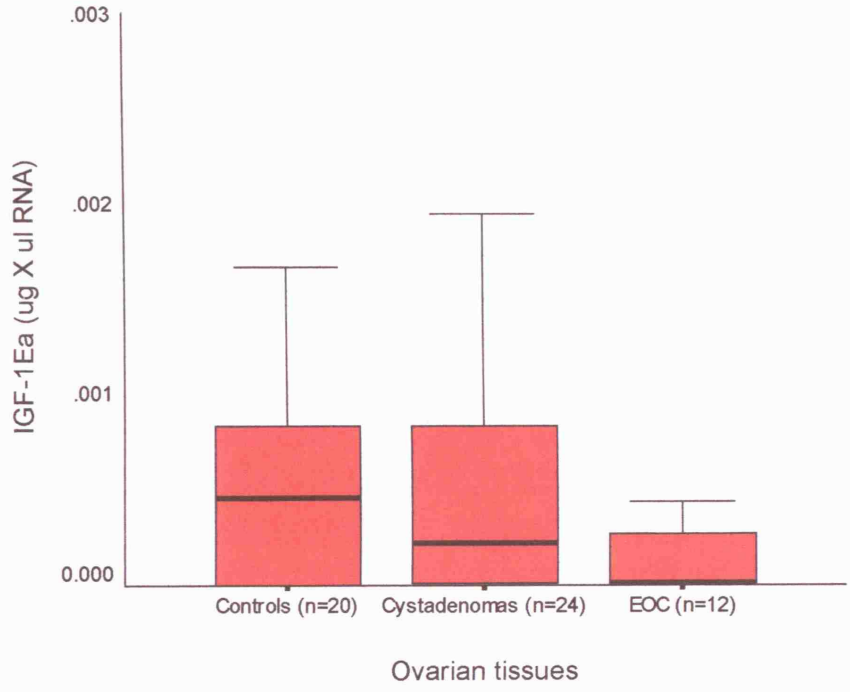
**Table 6.20 IGF-1Ea, IGF-1Ec and VEGF compared with MVD in ovarian tissues.**

### 6.4.8 RT-PCR RESULTS

RNA extraction was performed with concentrations ranging from 0.10 to 0.40 µg/µl.

#### 6.4.8.1 mRNA RESULTS FOR IGF-1Ea

Figure 6.17 shows the distributions of IGF-1Ea mRNA levels in different ovarian tissues and the mean values are as follows: controls = 7.10 X $10^{-4}$, cystadenomas = 5.43 X $10^{-4}$ and EOC = 1.85 X $10^{-4}$ µg X µl RNA. Although a decrease in IGF-1Ea levels was noted in EOC compared to controls or cystadenomas, this difference was not statistically significant (p=0.10 and 0.13 respectively).

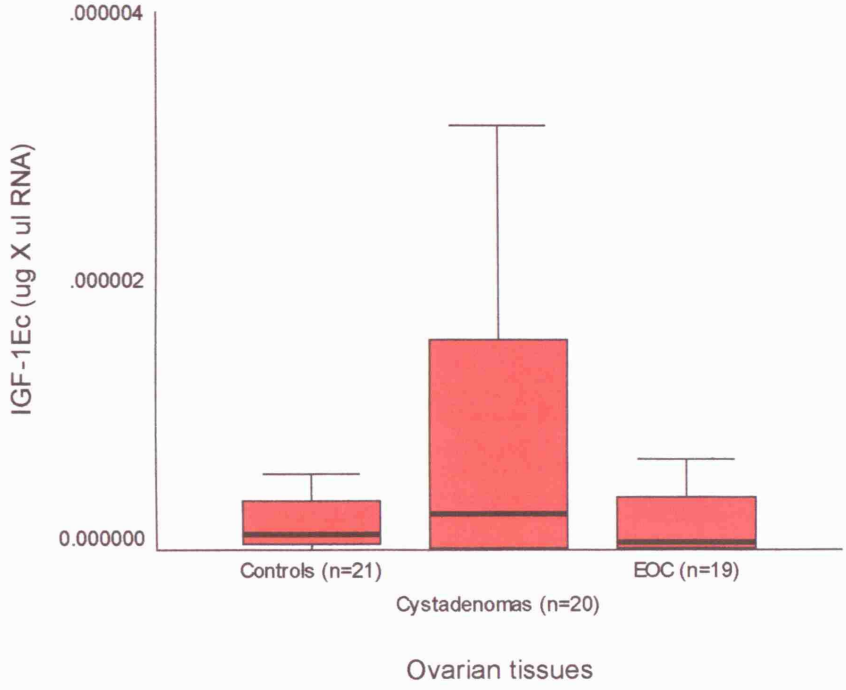



Bars and error bars represent mean and S.E.M respectively.
**Figure 6.17 Distributions of IGF-1Ea mRNAs in ovarian tissues.**

#### 6.4.8.2 mRNA RESULTS FOR IGF-1Ec

The distributions of the mean values of IGF-1Ec mRNA levels in the ovarian tissues are as follows: controls = 6.80 X $10^{-7}$, cystadenomas = 1.17 X $10^{-6}$ and

EOC = 3.03 X $10^{-7}$ μg X μl RNA (Figure 6.18). Although higher IGF-1Ec levels were noted in cystadenomas compared to controls or EOC, this difference was not statistically significant (p=0.06 and 0.42 respectively).




Bars and error bars represent mean and S.E.M respectively.
**Figure 6.18 Distributions of IGF-1Ec mRNAs in ovarian tissues.**

6.4.8.3 mRNA RESULTS FOR VEGF

The mean values of VEGF mRNA levels in the ovarian tissues are as follows: controls = 1.15 X $10^{-6}$, cystadenomas = 1.56 X $10^{-6}$ and EOC = 2.22 X $10^{-6}$ μg X μl RNA (Figure 6.19). Again although VEGF mRNA levels demonstrated an increase in EOC when compared with the control group (p=0.27), this difference was not significant, due to the wide variation in S.E.M.

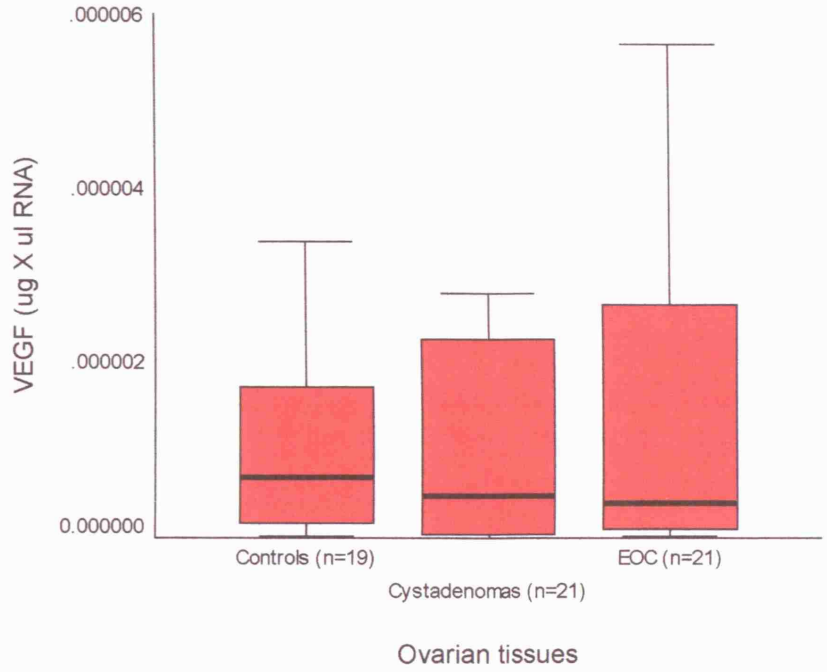



**Figure 6.19 Distributions of VEGF mRNAs in ovarian tissues.**

6.4.8.4 CORRELATION BETWEEN IGF-1Ea, IGF-1Ec AND VEGF

Table 6.21 shows the relationship of double markers for each tissue group based on matched samples. The control group showed several good correlations between these markers, however, in cystadenomas, no relationship was found. In contrast EOC showed a good correlation between IGF-1Ec and VEGF (p=0.04) but not between the other markers.

| | **IGF-1Ea vs IGF-1Ec** | **IGF-1Ea vs VEGF** | **IGF-1Ec vs VEGF** |
|---|---|---|---|
| **Controls** | 20 | 19 | 19 |
| **P value** | **0.007** | **0.014** | **0.005** |
| **Cystadenomas** | 20 | 21 | 20 |
| **P value** | 0.08 | 0.08 | 0.89 |
| **EOC** | 12 | 12 | 19 |
| **P value** | 0.07 | 0.08 | **0.04** |

**Table 6.21 Relationship between double markers in ovarian tissues.**

## 6.5 DISCUSSION

The incessant ovulation theory has been involved in the pathogenesis of EOC. This theory suggests that EOC arises from the surface epithelium or from epithelial inclusion cysts resulting from invagination of surface epithelium and entrapment of the surface epithelium within the ovarian stroma following the local trauma produced by ovulation. This theory supports epidemiological evidence showing that the risk of ovarian cancer increases with the number of ovulations women experience, hence the protective effect attributed to the use of oral contraception and pregnancy. Following, each ovulation, the ovary undergoes a process of repair of the surface epithelium. This repair process often leads to the formation of epithelial inclusion cysts suggesting that women at risk for ovarian cancer would have more epithelial inclusion cysts (Mittal, 2000; Westhoff et al, 1993). No attention has been paid to date to the repair mechanism itself as a contributing factor following ovulation injury.

### 6.5.1 IGF-1 STUDY

Table 6.22 shows the results of other studies with regard to IGF-1 in patients with ovarian tumours.

| Authors | Results |
|---|---|
| Bese and Nomir, 2001 | Pre-operative serum IGF-1 concentration showed an increase with chemotherapy. |
| Conover et al, 1998 | Epithelial carcinoma cells derived from fresh, untreated epithelial ovarian cancer specimens secrete IGF peptides and IGF binding proteins and possess type I IGF receptors. |
| Dursun et al, 2005 | Pre-operative serum IGF-1 levels were significantly higher in patients younger than 50 years old. |
| Gotlieb et al, 2006 | IGF-1 and IGF-2 were produced by 2 epithelial ovarian |

| | |
|---|---|
| | carcinoma cell lines (OVCAR-3 and OVCAR-4). |
| Karasik et al, 1994 | Highest IGF-1 concentration in serum from a group of patients with invasive ovarian cancers than in fluids from benign cysts or borderline tumours. |
| Lukanova et al, 2002 | Circulating IGF-1 was directly and strongly associated with ovarian cancer risk before age 55. |
| Shah et al, 1994 | A significant decrease in IGF-1 concentration in the serum of women with ovarian cancers was found compared to healthy women. |
| Shen et al, 2004 | IGF-1 stimulates KCl co-transport, necessary for proliferation and invasion of ovarian cancer cells. |
| Waksmanski et al, 2001 | IGF-1 concentrations decreased in the serum of postmenopausal women with ovarian tumours |
| Yee et al, 1991 | IGF-1 mRNA was detected in tumour specimens and in 3 ovarian cancer cell lines. |
| Yee et al, 1994 | IGF-1 mRNA was detected in ovarian cancer cells contained in malignant ascites but it was not detected in breast cancer cells. |

**Table 6.22 Studies of IGF-1 in ovarian cancers.**

From the table above, the majority of studies of IGF-1 has analysed either the mRNA expression of IGF-1 or IGF-1 concentrations in serum samples. Some serum studies reported a decrease in IGF-1 levels in patients with ovarian carcinomas compared to serum from benign cystadenomas (Shah et al, 1994; Waksmanski et al, 2001); however other studies report an increase in IGF-1 serum levels during the progression of EOC (Karasik et al, 1994; Lukanova et al, 2002). As a result of these conflicting findings, it is clear that measuring IGF-1 levels in serum samples of ovarian cancer patients will not be appropriate as a pre-operative diagnostic tool. This is the first study to analyse IGF-1 splice variants

protein and gene expression in EOC and to understand its mechanisms related to VEGF regulation and repair.

### 6.5.2 IGF-1Ea

Using IHC, results showed that the expression of IGF-1Ea is significantly higher in EOC than in benign cystadenomas and normal ovarian tissues, ($P<0.001$). However, no significant correlation was found between IGF-1Ea protein levels and tumour histological parameters such as subtype, tumour grade, or stage of the disease. Such findings are consistent with the study conducted by Karasik et al (Karasik et al, 1994) with the only difference being that IGF-1 was analysed in serum levels.

In contrast to the IHC results, RT-PCR showed that IGF-1Ea mRNA levels are lower in EOC versus the control group ($1.85 X10^{-4}$ versus $7.10 X 10^{-4}$). Although this difference is not statistically significant, this decrease in IGF-1Ea mRNA is probably a reflection of the instability of mRNA of this splice variant. These findings are similar to related studies showing a decrease in IGF-1 mRNA at the transition from normal epithelium to proliferative cancer, such as in thyroid and breast cancers (Heffelfinger at al, 1999; Vesely et al, 2004).

### 6.5.3 IGF-1Ec

In this study, IGF-1Ec, the splice variant responsible for satellite cell activation required for protein synthesis during repair of damaged muscle fibres, does not show any significant difference in protein expression between normal and malignant ovarian tissue or mRNA expression or between histopathological features such as tumour subtype, grade and stage. However, IGF-1Ec mRNA and

protein expression are overexpressed in the benign cystadenomas compared to EOC and normal ovaries, suggesting that the role of IGF-1Ec in benign cystadenomas (and may be in benign epithelial tumours in general) may reflect a repair activity in these tumours. Further studies analysing IGF-1Ec protein expression in inclusion cysts (which may be the site of tumour initiation) of post-menopausal ovaries would be valuable to support the ovulation or repair hypothesis. An understanding of the signalling pathways involved in the IGF-1Ea/Ec action may bring a insight on the role of these proteins in ovarian repair and proliferation.

### 6.5.4 VEGF

In this study, VEGF mRNA expression shows an increase from normal and benign cystadenomas to EOC; however this was not significant. Similar findings were reported by several studies (Sowter et al, 1997; Fujimoto et al, 1998; Wang et al, 2002). In addition, these data correlate with VEGF protein expression which was also highly expressed in EOC compared to benign cystadenomas or controls (see Chapters 3 and 4).

### 6.5.5 ASSOCIATION BETWEEN IGF-1 AND VEGF

Despite the differences in cellular location of VEGF and IGF-1 protein expression in the ovarian tissues, IHC results revealed that EOC cases are more likely to be positive for VEGF and one or both IGF-1 splice variants (48.6 % positive for VEGF and IGF-1Ea, 42.9 % for VEGF and IGF-1Ec and 34.3 % for VEGF and both splice variants) compared to controls or benign cystadenomas. The only significant difference was observed in IGF-1Ea and VEGF expression between

EOC and controls or benign cystadenomas (p=0.0001 and 0.0001 respectively). Similarly, RT-PCR results showed that both IGF-1Ea and VEGF mRNA levels are higher in EOC versus controls and cystadenomas, though not significant. These findings suggest that it is more likely for VEGF to be directly regulated by IGF-1Ea in malignant tumours, and that VEGF levels in EOC may be related to IGF-1 expression. It may be that IGF-1 induces VEGF mRNA and increases its protein production by both an increase in the transcriptional rate of the VEGF gene and the stability of mRNA (Warren et al, 1996). Studies conducted by Abd El Aal et al (2005) measuring serum VEGF and IGF-1 in patients with a pathological ovarian condition (PCOS) found a significant increase of VEGF levels with an increase in IGF-1 in those women with PCOS compared to controls. However, the exact source of the VEGF was not clarified and as shown in chapter 5, a major source of VEGF is platelets which may be a confounding factor.

At tissue level, only one study has examined IGF-1 and VEGF correlations in cancer. This was in colorectal tissue, in which the authors found significant differences in VEGF gross mRNA levels and IGF-1 mRNA in colorectal cancers compared with controls. However, this study suffers from the criticism that co-localisation of these two proteins at cellular level was not examined.

### 6.5.6 ASSOCIATION BETWEEN IGF-1 AND MVD

An increase in tissue vascularisation is often attributed to numerous processes including tissue remodelling, angiogenesis and vasculogenesis (Rivard and Isner, 1998). The role of each of these processes and their regulation in invasive cancer are still poorly understood. Even less well understood are the mechanisms of increased tissue vascularisation in pre-invasive tumour formation. Several studies

have found an increase in tissue vascularisation in primary ovarian pathologies that correlated with histological features such as tumour stage of the disease (Abulafia et al, 2000). In this study, the results fail to show any correlation between IGF-1 splice variants and MVD. However, there was a tendency for EOC positive for these splice variants to show lower MVD values than their corresponding negative counterparts. This may suggest that these splice variants regulate VEGF expression but may not be involved in the vascularisation of these tumours.

## 6.6 CONCLUDING RESULTS

In summary, this thesis has examined tissue and mRNA levels of IGF-1 splice variants in relation to VEGF. Results showed;

1. A tendency for IGF-1Ec protein levels to be higher in benign cystadenomas possibly associated with a repair mechanism, and a significant increase of IGF-1Ea tissue expression in EOC compared to benign cystadenomas or controls, that may be related to IGF-1 protein synthesis leading to tumour proliferation.
2. Correlations of mRNA levels between IGF-1Ec and VEGF in EOC. However the examination of co-localisation of these two proteins on the same section of normal and neoplatic ovarian tissues showed that although sections may be positive for mRNA protein, this does not imply a direct regulation. In fact VEGF and IGF-1Ea or IGF-1Ec immunolocalisation are found in separate areas. This is further supported by the MVD results showing no correlation between MVD and IGF-1 splice variants.

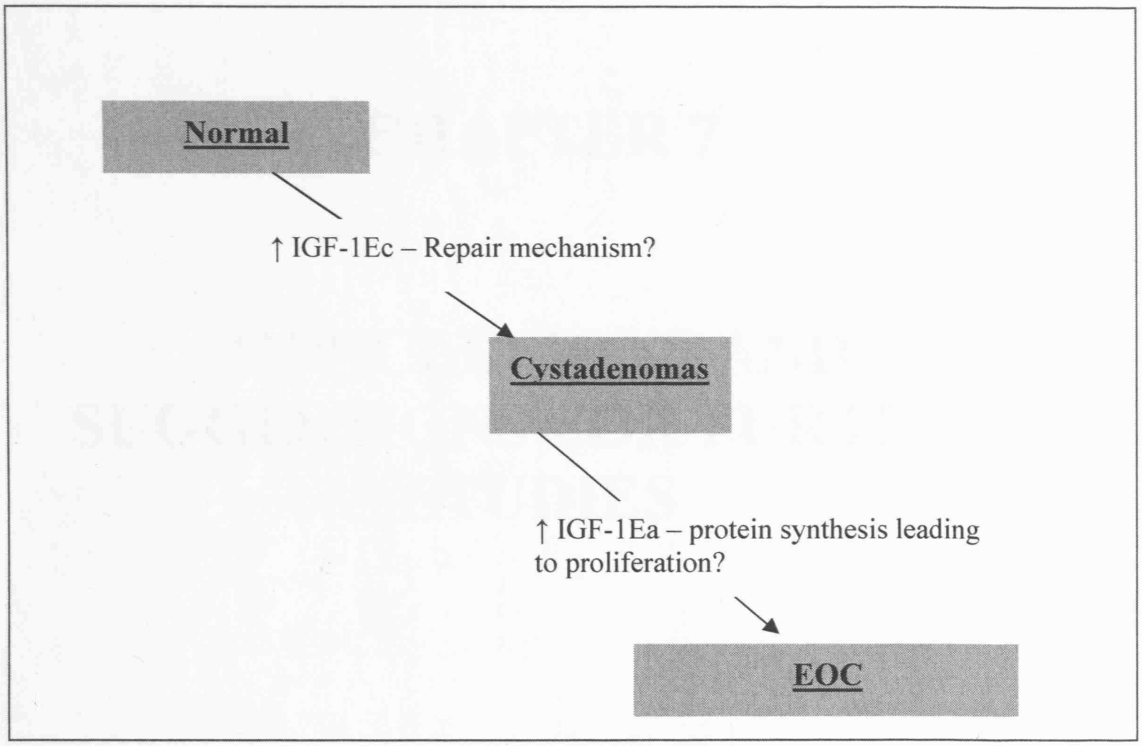



**Figure 6.20 Involvement of IGF-1 splice variants in EOC.**

# CHAPTER 7

# CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

## 7.1 CONCLUSIONS

To date, this thesis is the first study that has focussed on the molecular interplay between several VEGF members (VEGF itself, VEGF-C, VEGFR-1 and VEGFR-2), microvessel density (MVD), TP and IGF-1, and how these markers might contribute to a better understanding of the molecular pathways underlying angiogenesis / lymphangiogenesis during the formation of EOC. In the literature, a number of histopathological parameters such as the recognition of histopathological subtypes of ovarian carcinoma (serous, mucinous, endometrioid, clear cell or mixed subtype), grade of the disease and tumour stage have been used to predict the aggressiveness of EOC in the patient. However, no specific blood or tissue molecular marker of clinical diagnosis for EOC has been found.

A series of studies have looked at the correlation between VEGF and MVD, and between VEGF and its receptors in other types of human tumour, along with the interaction of these markers with other growth factors. A common picture of all these studies on angiogenesis was that VEGF has the most important role in the molecular pathways underlying tumour growth and angiogenesis. On the other hand, a correlation approach between different markers of angiogenesis in other tumours has shown that tumour progression does not involve VEGF alone, but also the interaction with other angiogenic factors, particularly VEGF-C and TP, thus showing the complexity of VEGF involvement in tumour proliferation.

The aim of this study was to identify additional prognostic parameters in EOC and potentially pre-malignant ovarian lesions, and to understand the potential mechanisms of angiogenesis / lymphangiogenesis in EOC progression. A series of laboratory-based research techniques, including immunohistochemistry, ELISA and real-time quantitative RT-PCR were performed to analyse the molecular

markers of angiogenesis and lymphangiogenesis (VEGF, VEGF-C, VEGFR-1, VEGFR-2, TP and MVD). IGF-1 isoforms involved in cell proliferation, repair and angiogenic regulation were also analysed.

This thesis attempted to answer the following questions:

1. Are there molecular differences, at the tissue and fluid level, and protein and mRNA levels, of these angiogenic markers between EOC and potentially pre-malignant ovarian lesions?
2. Can such molecular markers be of prognostic value for early detection of malignant ovarian lesions and do they correlate with other parameters in EOC such as histological subtype, tumour stage and disease grade?
3. Could the serum levels be useful to determine the detection and progression of potentially pre-malignant ovarian conditions to EOC?

Are there molecular differences, at the tissue and fluid levels, and protein and mRNA levels, of these angiogenic markers between EOC and potentially pre-malignant ovarian neoplasms?

From Table 7.1, VEGF expression at both protein and mRNA levels showed an increase from benign cystadenomas to EOC, suggesting that VEGF in EOC may be the main factor among all these angiogenic proteins in ovarian tumorigenesis and its progression may be related to a late event (i.e. after invasion has occurred) in ovarian cancer progression.

TP appeared to play a role in the development of both serous and mucinous EOC from their corresponding benign cystadenomas; however, in borderline tumours (see Figure 4.13), it appeared to be more influential in the earlier stages leading to the formation of these potentially malignant tumours rather than the formation of

the corresponding EOC subtypes. This is in keeping with current theories that not all EOC pass through a borderline stage.

The presence of higher VEGF-C levels in the formation of benign ovarian neoplasms compared to EOC reflects the involvement of lymphangiogenesis mainly during the earlier neoplastic changes involved in the pre-malignant ovarian lesions.

In addition, the results of this study demonstrate that both VEGF and TP are highly expressed in ovarian endometriosis suggesting that both growth factors may be crucial to the stepwise sequence leading to endometrioid-clear cell borderline ovarian tumours or directly to endometrioid –clear cell EOC.

| | **Controls** | **Potentially pre-malignant ovarian lesions** | | | **EOC** |
|---|---|---|---|---|---|
| | | **Benign cystadenomas** | **Endometriosis** | **Borderline tumours** | |
| **VEGF** | 16.7 | 27.8 | 85.2* | 40 | 68.3* |
| **VEGF mRNA** | 1.15 X 10-6 | 1.56 X 10-6 | - | - | 2.22 X 10-6* |
| **VEGF-C** | 0 | 87.5* | na | 90* | 68.3* |
| **VEGFR-1** | 100 | 80 | 88.9 | 100 | 92.5 |
| **TP** | 14.3 | 37.5 | 100* | 80* | 76.9* |
| **MVD (HVD)** | 80.8 ± 19.9 | 68.2 ± 18.3 | 56.0 ± 31.6* | 54.8 ± 22.0* | 57.8 ± 23.3* |
| **MVD (AVD)** | 67.1 ± 16.1 | 60.1 ± 16.5 | 44.7 ± 23.8* | 47.2 ± 21.5* | 47.0 ± 17.8* |

*Significant versus controls, $p<0.05$. Protein levels expressed as % of positive cases, mRNA levels expressed as (μg X μl RNA) and mean MVD ± SD.

**Table 7.1 Summary of the protein and mRNA results of the angiogenic markers.**

Secondly, at the fluid level, circulating levels of VEGF and VEGFR-2 were measured in EOC patients versus patients with benign ovarian cystadenomas or controls. Table 7.2 shows that VEGF serum levels increase significantly from benign ovarian cystadenomas to EOC cases ($p=0.001$). A significant increase in

platelet counts was also found from benign cystadenomas to EOC (p=0.04). However, it is not clear whether the increased VEGF causes the increase in platelet counts or whether the increased platelets release more VEGF, since platelets are known to produce VEGF themselves.

| Molecular marker (pg/ml) | Samples | Controls | Benign cystadenomas | EOC |
|---|---|---|---|---|
| VEGF | serum | 257 | 248 | 598* |
| | cyst fluids | - | 966 | - |
| | ascitic fluids | - | - | 3250 |
| VEGFR-2 | serum | 8287 | 8375 | 7898 |
| Platelet count | serum | 289 | 241 | 335 |

*Significant versus controls, as $p<0.05$.

**Table 7.2 VEGF, VEGFR-2 and platelet levels in ovarian fluids.**

Thirdly, the results of this study demonstrate elevated VEGF concentrations in ascitic fluids from EOC patients and cystic fluids from benign lesions compared to serum samples, suggesting a gradient effect, and confirming the belief that VEGF is produced in ovarian lesions, and then excreted into the circulation.

Finally, a comparison between controls and EOC shows higher levels of IGF-1Ea protein levels but lower levels of IGF-1Ec (see Table 7.3). This suggests that IGF-1 splice variants may have different functions in ovarian pathogenesis and IGF-1Ec expression may precede IGF-1Ea expression in ovarian cancer. Like IGF-1Ea, VEGF shows an increase from normal ovarian tissues to EOC and this difference was significant. This trend of increased expression by both markers towards malignant transformation of tissues suggests that VEGF may be under the regulation of IGF-1Ea.

A comparison at the mRNA level of the IGF-1 splice variants showed higher IGF-1Ec mRNA values in benign cystadenomas compared to EOC. This trend is reflected when IGF-1Ec protein levels were analysed (more benign cystadenomas expressed IGF-1Ec compared to EOC). Analysis of IGF-1Ea mRNA levels showed lower IGF-1Ea mRNA values in EOC compared to controls; however, at protein levels, IGF-1Ea is higher in EOC compared to controls. The latter finding suggests that IGF-1Ea mRNA may be less stable, possibly due to its involvement in the regulation of VEGF.

| **Molecular levels** | **Molecular markers** | **Controls** | **Benign cystadenomas** | **EOC** |
|---|---|---|---|---|
| **Tissue Protein (% of positive cases)** | **IGF-1Ea** | 33.3 | 26.1 | 82.5 |
| | **IGF-1Ec** | 61.1 | 78.3 | 54.3 |
| | **VEGF** | 16.7 | 27.8 | 68.3* |
| **Tissue mRNA (mean values, µg/µl)** | **IGF-1Ea** | 7.10 X 10-4 | 5.43 X 10-4 | 1.85 X 10-4 |
| | **IGF-1Ec** | 6.80 X 10-7 | 1.17 X 10-6 | 3.03 X 10-7 |
| | **VEGF** | 1.15 X 10-6 | 1.56 X 10-6 | 2.22 X 10-6* |

*Significant versus controls, as $p<0.05$.

**Table 7.3 IGF-1 splice variants and VEGF in ovarian tumours.**

These overall results therefore show that there are molecular differences at the tissue and fluid levels and, protein and RNA levels of these angiogenic markers between EOC and potentially pre-malignant ovarian lesions compared to controls. Among these markers, VEGF is the growth factor which is mainly expressed in EOC progression. In addition, EOC and potentially pre-malignant ovarian conditions such as endometriosis and borderline tumours demonstrate some similarities in the immunophenotypes of various angiogenic markers.

Can such molecular markers be of prognostic value for potentially pre-malignant lesions and are they correlated with other parameters in EOC such as high grade / high stage of the disease?

When comparing normal ovarian tissue with potentially pre-malignant lesions (benign cystadenomas, endometriosis and borderline tumours), two common molecular markers are found to be up-regulated. They are VEGF and TP. The similarity in expression of these two molecular markers suggests that they may reflect a common pathway underlying the early angiogenic changes occurring in normal tissue to become potentially pre-malignant lesions.

A closer examination of these molecular markers among the different EOC subtypes, showed higher expression of VEGF and TP in EOC endometrioid and clear cell subtype and higher VEGF-C expression in the mucinous subtype. Different angiogenic pathways may therefore be suggested to regulate the biology of these histological ovarian tumour types.

When MVD was measured in higher stages / grades of EOC, no differences in HVD (high vessel density) or AVD (average vessel density) were found when compared with those of early stages or lower grades. These molecular markers (VEGF, VEGF-C, VEGFR-1, TP and MVD) although combined with these histopathological parameters (subtype, stage and grade), therefore do not bring enough additional value to the prognostic aspect of EOC.

Could the serum levels be useful to determine the detection and progression of potentially pre-malignant ovarian neoplasms to malignant EOC?

Women aged more than 51 years old - regardless of the type of ovarian lesions with which they present (benign or malignant) - tend to show high levels of

sVEGF, regardless of the difference in sVEGFR-2 levels. This finding may reflect a common trend of the levels of VEGF occurring in women aged more than 51; hence the evaluation of VEGF in serum of women aged more than 51 could be useful to identify those more at risk of developing EOC.

## 7.2 FUTURE WORK

In the last 40 years, thousands of research articles have been published to describe the role of angiogenesis (sprouting of new blood vessels from an existing vascular network) in the onset and/or progression of such diseases as cancer, rheumatoid arthritis, psoriasis and diabetes. There are currently increasing numbers of angiogenesis assays being described *in vitro* and *in vivo*.

The *in vitro* models of angiogenesis have focused predominantly on migration, proliferation and tubule formation by endothelial cells in response to exogenous inhibitory or stimulatory agents. Assays such as the cell cycle analysis, the BrDU assay (Gomez and Reich, 2003), Tritiated thymidine (Freshney, 1994) and the MTT (Denizot and Lang, 1986; Wemme et al, 1982) measure cell proliferation. The Boyden chamber (Falk et al, 1980), the 'phagokinetic track' (Zetter, 1987) and the 'wound healing' assays (Auerbach et al, 1991) measure cell migration. Specific assays to measure differentiation are the matrix assays (Fawcett, 1994), the 3D gel (Gagnon et al, 2002) and co-cultures of human fibroblasts and endothelial cells (Bishop et al, 1999). These mimic the *in vivo* situation and rely on fibroblasts secreting the necessary matrix components that act as a scaffold for tubule formation where formation of tube like structures are formed and measured.

In vivo assays include the corneal angiogenesis assay (Gimbrone et al, 1974), the chick chorio allantoic membrane (Nguyen et al, 1994; Ribatti et la, 1996), the dorsal air sac model (Oikawa et al, 1997), the chamber assays (Jain 1997), the tumour models (Sato et al, 1986) and the zebrafish assay (Rubinstein, 2003). Some of these assays have significance for screening, while others are used primarily in studies of dosage-effects, molecular structure activities and the combined effects of two or more agents on angiogenesis.

However, these current methods for assaying angiogenesis in vitro and in vivo have their limitations. *In vivo* tests tend to be more difficult to set up, and frequently require surgical skills thereby limiting the number of tests that can readily be performed. Currently new methods for imaging vessels and for image analysis are emerging that may help provide quantification of both *in vivo* and *in vitro* experiments. Therefore it will be essential to quantify the effects of a particular test substance such as VEGF or TP on the process of angiogenesis by using more than one in vitro assays and using different sources of endothelial cells and then follow these with more than one in vivo assay to ensure that the results seen in vitro translate across to the in vivo state where other cells and extra-cellular matrix proteins are really involved in the process of angiogenesis.

IGF-1Ea protein seems to show the same pattern of expression as VEGF at the tissue level (i.e. high in EOC). The analysis of IGF-1 splice variants in endometriosis and borderline tumours would be interesting to investigate as VEGF was found to be high in endometriosis but low in borderline tumours. If the same trend was to be found in these potentially pre-malignant ovarian lesions,

IGF-1Ea would provide a better understanding of its regulation on VEGF and possibly its role as a repair mechanism of ovulation.

Another angiogenic marker, placental growth factor (PlGF) has recently been shown to affect only angiogenesis in disease without affecting quiescent vessels in healthy organs (Luttun et al, 2004). The assessment of PlGF expression could therefore allow further insight in the angiogenic events of EOC and its correlation with VEGF could produce further information on the diagnostic aspect.

Recently, endoglin (CD105), a member of the transforming growth factor-1 receptor complex, has been shown to be a useful marker to identify tumour angiogenesis in certain human cancers such as endometrium and oesophagus (Saad et al, 2003; 2005). The advantage of endoglin is that it binds preferentially to the activated endothelial cells during angiogenesis (Westphal et al, 1993; Seon et al, 1997) and thus it is potentially a more specific marker for tumour neovascularisation. From this thesis, the measurement of MVD alone or combined with VEGF or TP does not appear to be associated with angiogenesis and so further investigation using endoglin would further clarify this issue.

Recently, some studies have reported that tumour suppressor genes such as p53, PTEN and VHL increase VEGF, by inducing HIF-1 activity, in ovarian cancer cells (Horiuchi et al, 2002; Mukhopadhyay and Datta, 2004; Fang et al, 2005). Using laser capture microdissection to select tumour cells in EOC from either fresh or frozen tissues and performing mutational analysis on these tissues would

allow the comparison of p53, PTEN and VHL protein expression with VEGF in the progression of EOC.

There is convincing evidence for other tumours (of the cervix, endometrium and colon) and their corresponding pre-invasive lesions that angiogenesis is an important event, and a molecular marker of tumour behaviour. This study has looked at various ovarian pathogenesis for differences in angiogenic markers. Some of the differences have been statistically significant but their biological importance remains uncertain. It seems that greater numbers must be analysed to find clinically or therapeutically important differences.

# APPENDIX

## IMMUNOHISTOCHEMISTRY: REAGENTS AND BUFFERS

1% (v/v) Acid-alcohol
1400 ml ethanol
580 ml distilled water
20 ml concentrated HCl

Acetone (BDH)

3-Aminopropyltriethoxysilane solution -APES (Sigma-Aldrich A3648)
294 ml of acetone with 6ml APES
Keep APES at 4°C and dilute in acetone just before use.
APES coating for slides
1. Wash in detergent for 20 minutes.
2. Rinse in distilled water and dehydrate in methanol.
3. Dry at room temperature.
4. Drop in APES solution in fume cupboard, rinse in distilled water.
5. Dry overnight at 37°C.

Blueing solution
1. Add 5g disodium tetraborate to 1 litre distilled water. 0.5% (w/v) final concentration.

Bovine serum albumin (Fraction V, Sigma A-2153)

Citric acid monohydrate (Merck, 'Analar' 100813M)

DPX mountant (Merck 360294H)

Diaminobenzidine–DAB (Sigma-Aldrich, D-5637)

Disodium hydrogen phosphate anhydrous (Sigma-Aldrich, S0876)

Disodium tetraborate (Merck, 102674E)

Ethanol (Hayman 'Absolute alcohol 100')

Formaldehyde
Formol saline
40% formaldehyde 100ml
Sodium chloride 9g
Water 900ml
Neutral buffered formaldehyde
40% formaldehyde 100ml
Distilled water 900ml
Sodium dihydrogen phosphate monohydrate 4g
Disodium hydrogen phosphate anhydrous 6.5g

Haematoxylin and Eosin

Hydrocholoric acid-concentrated (Merck, 'Analar' 101250D)

Hydrogen peroxide solution (Merck, 'Analar' 101284N)
1. Add 10ml 30% hydrogen peroxide to 90 ml distilled water.

Imidazole Solution (Merck, GPR 285466K)

1. Add 0.681g imidazole to 100ml distilled water.
2. Store at 4°C.
3. 0.1M imidazole solution.

Industrial methylated spirit

Mayers Haemalun 'Gurr' (BDH 350604T)

Methanol (Merck, 'Analar' 101586B)

NaCl (Merck, 'Analar' 1913833X)

Normal Goat Serum (Dako X0907)

Normal Rabbit Serum (Dako, X0902)

Fresh Phosphate Buffered Saline (Sigma Aldrich, P4417)

1. Dissolve 1 tablet in 200ml distilled water.

Phosphate Buffered saline (ICN 17-604-20)

1. Dissolve 5 PBS powder sachets in 5l distilled water. pH 7.4

Protease XXIV (Sigma Aldrich P8038)

Protease solution for protease digestion:

1. Dissolve 37.5mg of protease XXIV in 200ml fresh PBS at 37°C.
2. Leave tissue sections for 7 minutes.

Sodium Citrate Buffer

1. Weigh out 2.1g citric acid monohydrate
2. Add 950 ml distilled water
3. Add 13ml 2M NaOH. Make up to 1l.
4. Adjust pH 6.0. Store at 4°C.
5. 0.01M Sodium citrate (pH 6.0 at 25°C).

Streptavidin-biotin complex duet kit (Dako K0492)

Tris [ Tris (hydroxymethy) methylamine] (Merck 'Aristar' 452054C)

Tris buffered saline (TBS)

1. Weigh out 43.83g NaCl and 30.6g Tris base.
2. Make up to 5l distilled water.
3. Add ~35ml of concentrated HCl, ensure pH is at 7.6.
4. Store at 4°C.
5. 0.05M Tris-HCl, 0.15M NaCl, pH 7.6.

Xylene (Merck 'Analar'102936H)

## IMMUNOHISTOCHEMISTRY: PRIMARY AND SECONDARY ANTIBODIES

| PRIMARY ANTIBODIES | | | | | |
|---|---|---|---|---|---|
| **Antigen** | **Source** | **Species** | **Clone** | **Isotype** | **Positive control** |
| IGF-1Ea | Santa Cruz Sc 9013 | Rabbit polyclonal | H-70 | IgG | Muscle |
| IGF-1Ec | Gift from Prof Goldspink* | Rabbit polyclonal | - | IgG | Muscle |
| Human TP | Lab Vision MS-499-R7 | Mouse monoclonal | P-GF.44C | $IgG_1$ | Breast carcinoma |
| Human VEGF | R&D MAB293 | Mouse monoclonal | 26503.111 | $IgG_{2B}$ | Placenta |
| Human VEGF-C | Santa Cruz Sc-1881 | Goat polyclonal | A-3 | IgG | Placenta |
| Human VEGFR-1 | Santa Cruz Sc-6251 | Mouse monoclonal | C-20 | $IgG_1$ | Colon carcinoma |
| Human vWF | Dako M0616 | Mouse monoclonal | F8/86 | $IgG_1, \kappa$ | Placenta |

* Department of Surgery RFH NHS Trust Hospital (ref: Cortes et al, 2005).

| SECONDARY ANTIBODIES | | |
|---|---|---|
| **Species** | **Conjugate** | **Source** |
| Rabbit anti-mouse poly | Biotin | Dako E0354 |
| Rabbit anti-mouse IgM poly | Biotin | Dako E0465 |
| Goat anti-rabbit poly | Biotin | Dako E0432/K0492 |

## ELISA: REAGENTS AND BUFFERS

Human VEGF ELISA kit (R&D Systems, DVE00)

Assay Diluent RD1W (895117): 11ml of a buffered protein base with preservatives.
Calibrator Diluent RD6U (895148): 21ml of animal serum.
Color Reagent A (895000): 12.5ml of stabilised hydrogen peroxide.
Color Reagent B (895001): 12.5ml of stabilised tetramethylbenzidine.
Stop solution (895032): 6ml of 2N sulphuric acid.
VEGF Conjugate (890219): 21ml of polyclonal antibody against VEGF conjugated to horseradish peroxidase.
VEGF microplate (890218): 96 well polystyrene microplate coated with a mouse monoclonal antibody against VEGF.
VEGF Standard (890220): 2000 pg/vial of recombinant human $VEGF_{165}$ in a buffered protein base
Wash Buffer Concentrate (895003): 21ml of a 25 fold concentrated solution of buffered surfactant with preservative.

Human VEGFR-2 ELISA kit (R&D Systems, DVR200)

Assay Diluent RD1W (895117): 11ml of a buffered protein base with preservatives.
Calibrator Diluent RD6-31 (895323): 21ml/vial of animal serum.
Color Reagent A (895000): 12.5ml of stabilised hydrogen peroxide.
Color Reagent B (895001): 12.5ml of stabilised tetramethylbenzidine.
Stop solution (895032): 6ml of 2N sulphuric acid.
VEGFR-2 Conjugate (890931): 21ml of polyclonal antibody against VEGFR-2 conjugated to horseradish peroxidase.
VEGFR-2 microplate (890930): 96 well polystyrene microplate coated with a murine monoclonal antibody against VEGFR-2.
VEGFR-2 Standard (890932): 50ng/vial of recombinant human VEGFR-2 in a buffered protein base
Wash Buffer Concentrate (895003): 21ml of a 25 fold concentrated solution of buffered surfactant with preservative.

## RNA EXTRACTION: REAGENTS, BUFFERS AND PROTOCOLS

RNeasy RNA extraction kit (Qiagen 74104)

β-Mercaptoethanol or β-ME ( ): 10µl β-ME added to 1ml Buffer RLT.
Buffer RLT (R20): contains guanidine thiocyanate
Buffer RPE (supplied as a concentrate); 4 volumes of ethanol (96-100%) added to obtain a working solution.
Buffer RW1 (R10): contains ethanol.
RNeasy mini spin columns.

1. Excise the tissue sample and store at -70°C. When required, the frozen tissue is placed on dry ice and a portion is cut on a clean surface, on dry-ice.
2. The amount of tissue to be used is then determined by weighing (do not use more than 30mg).
3. Place excised tissue into a suitably vessel for disruption and homogenisation.
4. Disrupt the tissue and homogenise the lysate in ~600µl Buffer RLT, using a conventional rotor-homogeniser (e.g. Polytron).
5. Centrifuge the lysate for 3 min at 8000g and this lysate will be used in subsequent steps.
6. Add 1 volume of 70% ethanol to the cleared lysate and mix immediately by pipetting.
7. Transfer up to 700µl of sample to an RNeasy mini spin column placed in a 2ml collection tube (supplied).
8. Add 700µl Buffer RW1 to the RNeasy mini spin column. Close lid gently and centrifuge for 15 seconds at 8000g to wash the spin column membrane. Discard flow-through.
9. Add 500µl Buffer RPE to RNeasy mini spin column. Close lid gently and centrifuge for 15 seconds at 8000g. Discard flow-through.
10. Add 500µl Buffer RPE to RNeasy mini spin column. Close lid gently and centrifuge for 2 minutes at 8000g to wash the spin column membrane. Discard flow-through.
11. Place the RNeasy mini column in a new 2ml collection tube and discard the old flow-through. Close lid gently and centrifuge for 1 minute at full speed.
12. Place the RNeasy mini column in a new 1.5ml collection tube (supplied). Add 50µl of 1% DEPC water or 50µl RNase-free water directly to the spin column membrane. Close lid gently and centrifuge for 1 minute at 8000g to elute the RNA.

## cDNA SYNTHESIS

Omniscript Reverse transcriptase kit (Qiagen 205111)

1. Thaw RNA template solution on ice,
2. Thaw primer solutions, 10x Buffer RT, dNTP Mix and RNase-free water at room temperature. After thawing, store on ice.
3. Mix each solution by vortexing and centrifuge briefly to collect residual liquid.
4. Dilute RNase inhibitor to a final concentration of 10 units /µl in 1x Buffer RT. Mix by vortexing and centrifuge briefly for 5 seconds.
5. Prepare master mix as shown in table below

| **Component** | **Volume/reaction** | **Final concentration** |
|---|---|---|
| **Master mix** | | |
| 10x Buffer RT | 2µl | 1x |
| dNTP Mix (5mM each dNTP) | 2µl | 0.5 mM each dNTP |
| Oligo –dT primer (10µM) | 2µl | 1 µM |
| RNase inhibitor (10 units/µl) | 1µl | 10 units (per 20 µl reaction) |
| Omniscript reverse transcriptase | 1µl | 4 units (per 20 µl reaction) |
| RNase-free water | 1 µl | - |
| **Template RNA** (added at step 7) | Variable* | Up to 2µg (per 20 µl reaction) |
| Total volume | 20 µl | - |

6. Vortex master mix for no more than 5 seconds.
7. Add template RNA to individual tubes and vortex for 5 seconds. Centrifuge briefly (1 minute)
8. Incubate for 60 min at 37°C.
9. Transfer to 93°C for 5 min followed by rapid cooling on ice.
10. Store samples at -70°C or proceed with qRT-PCT.

## QUANTITATIVE REAL TIME RT-PCR (qRT-PCR)

QuantiTect™ SYBR® Green PCR kit (Qiagen 20413)

1. Thaw 2X Quantitect SYBR Green PCR Master, template DNA, primers, RNase –free water and MgCl2 stock solution.
2. Prepare master mix according to table below:

| **Component** | **Volume/reaction** | **Final concentration** |
|---|---|---|
| 2X Quantitect SYBR Green PCR | 10 µl | 1x |
| Primer U (upstream or forward) | 1 µl | 0.5 µM |
| Primer D (downstream or reverse) | 1 µl | 0.5 µM |
| RNase-free water | 6 µl | - |
| Template DNA (added at step 4) | 2 µl | 1 µg/reaction |
| **Total volume** | **20 µl** | |

3. Mix the Master mix thoroughly and dispense appropriate volumes into PCR capillaries.
4. Add template DNA to individual capillaries.
5. Program LightCycler as shown in table below:

| **Step** | Time | Temperature |
|---|---|---|
| **Heating** | 2 min | 50°C |
| **PCR**<br>Initial activation step | 15 min | 95°C |
| Denaturation step | 15s | 94°C |
| Annealing step | 20s | 58°C (for VEGF)<br>60°C (for IGF-1Ea and Ec) |
| Extension | 5-25s | 72°C |
| Cycle number | 45 (VEGF)<br>40 (IGF-1Ea)<br>40 (IGF-1Ec) | |

6. Place PCR capillaries in Lightcycler and start cycling program.

## PATIENTS' DETAILS AND IHC RESULTS

| NO | age | VEGF (fa) | VEGF-C (%) | VEGFR-1 (%) | TP (%) | AVDx200 | HVDx200 |
|---|---|---|---|---|---|---|---|
| NO1 | 51 | 17 | 0 | . | . | 55.35 | 66.15 |
| NO2 | 46 | 12 | 0 | . | . | 62.51 | 83.7 |
| NO3 | 45 | 3 | 0 | 50 | 0 | 64.8 | 71.55 |
| NO4 | 25 | 0 | 0 | 30 | 0 | 79.65 | 94.5 |
| NO5 | 51 | 7 | 0 | . | . | 89.1 | 110.7 |
| NO6 | 38 | 7 | 0 | . | . | 81 | 97.2 |
| NO7 | 47 | 5 | 0 | . | . | . | . |
| NO8 | 59 | 0 | 0 | 30 | 0 | 60.75 | 67.5 |
| NO9 | 74 | 1 | 0 | 60 | 50 | 67.5 | 71.55 |
| NO10 | 57 | 13 | . | . | 0 | 93.15 | 108 |
| NO11 | 49 | 8 | 0 | . | . | 72.5 | 98.55 |
| NO12 | 48 | 2 | 0 | 20 | 0 | 91.8 | 101.25 |
| NO13 | 54 | 8 | 0 | . | . | 36.45 | 43.2 |
| NO14 | 50 | 6 | . | 40 | 0 | 66.56 | 71.55 |
| NO15 | 40 | 10 | 0 | . | 0 | 54 | 63.45 |
| NO16 | 43 | 0 | 0 | . | 0 | 60.75 | 75.6 |
| NO17 | 62 | 0 | 2 | . | 20 | 55.35 | 63.45 |
| NO18 | 48 | 8 | 0 | | . | 81 | 114.75 |
| NO19 | 44 | 2 | 0 | | 0 | 44.55 | 56.7 |
| NO20 | 44 | 7 | 0 | | 0 | 49.95 | 67.5 |
| NO21 | 46 | 5 | . | | . | 51.71 | 72.9 |
| NO22 | 81 | 5 | 0 | | . | 54.41 | 59.4 |
| NO23 | 49 | 1 | 0 | | 0 | 81 | 91.8 |
| NO24 | 46 | 1 | 0 | | 0 | 89.1 | 106.65 |

NO = normal ovarian tissue (or controls), fa = focal areas.

| Cy | age | type | VEGF (fa) | VEGF-C (%) | VEGFR-1 (%) | TP (%) | AVD x200 | HVD x200 |
|---|---|---|---|---|---|---|---|---|
| Cy1 | 34 | muc-cyst | 5 | 60 | 40 | 5 | 47.25 | 47.25 |
| Cy2 | 24 | muc-cyst | 4 | 50 | 0 | 10 | 73.85 | 79.65 |
| Cy3 | 45 | ser-cyst | 2 | 50 | 0 | 0 | 44.55 | 49.95 |
| Cy4 | 44 | muc-cyst | 3 | 60 | 20 | 10 | 52.25 | 56.7 |
| Cy5 | 32 | ser-cyst | 3 | 50 | 50 | 5 | 52.25 | 56.7 |
| Cy6 | 61 | ser-cyst | 12 | 30 | 30 | 5 | 53.6 | 58.05 |
| Cy7 | 37 | muc-cyst | 1 | 50 | 30 | 20 | 56.36 | 85.05 |
| Cy8 | 38 | ser-cyst | 3 | . | . | . | . | . |
| Cy9 | 59 | muc-cyst | 0 | . | . | . | . | . |
| Cy10 | 47 | muc-cyst | 15 | 50 | 20 | 0 | 69.8 | 81 |
| Cy11 | 89 | ser-cyst | 1 | 0 | . | 0 | . | . |
| Cy12 | 31 | ser-cyst | 4 | 50 | 0 | 10 | 36.45 | 39.15 |
| Cy13 | 56 | muc-cyst | 4 | 50 | 10 | 0 | 49.01 | 64.8 |
| Cy14 | 50 | muc-cyst | 8 | 0 | 30 | 0 | 55.35 | 66.15 |
| Cy15 | 33 | muc-cyst | 11 | 70 | 30 | 10 | 93.15 | 101.25 |
| Cy16 | 39 | ser-cyst | 16 | 60 | 20 | 0 | 93.56 | 99.9 |
| Cy17 | 75 | muc-cyst | 10 | 50 | 20 | 10 | 61.56 | 70.2 |
| Cy18 | 51 | muc-cyst | 9 | 60 | 30 | 5 | 62.51 | 67.5 |

Cy = ovarian cystadenomas, fa = focal areas, muc = mucinous, ser = serous.

| Ems | age | VEGF (fa) | VEGFR-1 (%) | TP (%) | AVDx200 | HVDx200 |
|---|---|---|---|---|---|---|
| Ems1 | 39 | 20 | 30 | 80 | 124.2 | 162 |
| Ems2 | 47 | 10 | 30 | 80 | 90.45 | 128.25 |
| Ems3 | 46 | 19 | 50 | 10 | 54 | 70.2 |
| Ems4 | 43 | 17 | 40 | 20 | 27 | 33.75 |
| Ems5 | 37 | 9 | 50 | 25 | 41.85 | 55.35 |
| Ems6 | 46 | 7 | 40 | 80 | 28.35 | 29.7 |
| Ems7 | 41 | 19 | 60 | 10 | 24.3 | 29.7 |
| Ems8 | 43 | 10 | 50 | 20 | 41.85 | 48.6 |
| Ems9 | 36 | 13 | 40 | 10 | 58.05 | 62.1 |
| Ems10 | 43 | 25 | 50 | 50 | 27 | 29.7 |
| Ems11 | 48 | 40 | 30 | 75 | 45.9 | 70.2 |
| Ems12 | 38 | 13 | 10 | 75 | 35.1 | 43.2 |
| Ems13 | 48 | 6 | 50 | 50 | 45.9 | 51.3 |
| Ems14 | 49 | 19 | 40 | 25 | 33.75 | 41.85 |
| Ems15 | 44 | 12 | 20 | 80 | 37.8 | 43.2 |
| Ems16 | 36 | 18 | 20 | 50 | 49.95 | 60.75 |
| Ems17 | 47 | 8 | 40 | 50 | 74.25 | 82.35 |
| Ems18 | 47 | 42 | 30 | 20 | 39.15 | 45.9 |
| Ems19 | 42 | 22 | 0 | 50 | 21.6 | 22.95 |
| Ems20 | 50 | 11 | 5 | 80 | 5.4 | 10.8 |
| Ems21 | 73 | 16 | 20 | 10 | 33.75 | 37.8 |
| Ems22 | 43 | 11 | 20 | 30 | 35.1 | 49.95 |
| Ems23 | 42 | 11 | 30 | 50 | 32.4 | 45.9 |
| Ems24 | 38 | 16 | 20 | 50 | 32.4 | 37.8 |
| Ems25 | 39 | 20 | 40 | 50 | 74.25 | 86.4 |
| Ems26 | 48 | 47 | 50 | 20 | 47.25 | 67.5 |
| Ems27 | 54 | 37 | 5 | 50 | 45.9 | 63.45 |

Ems = endometriosis, fa = focal areas.

| Bot | age | type | VEGF (fa) | VEGF-C (%) | VEGFR-1 (%) | TP (%) | AVDx200 | HVDx200 |
|---|---|---|---|---|---|---|---|---|
| Bot1 | 57 | ser-bot | 2 | 50 | 50 | 30 | 11 | 17.55 |
| Bot2 | 73 | ser-bot | 15 | 0 | 50 | 5 | 49.3 | 78.3 |
| Bot3 | 29 | ser-bot | 19 | 50 | 50 | 50 | 44.7 | 67.5 |
| Bot4 | 53 | ser-bot | 33 | 20 | . | 10 | 45 | 70.2 |
| Bot5 | 52 | muc-bot | 2 | 30 | 70 | 10 | 33 | 51.3 |
| Bot6 | 35 | muc-bot | 2 | 30 | . | 30 | 21.6 | 40.5 |
| Bot7 | 55 | muc-bot | 4 | 20 | 40 | 80 | 24.3 | 44.55 |
| Bot8 | 75 | muc-bot | 5 | 30 | . | 20 | 25.3 | 37.8 |
| Bot9 | 55 | endo-bot | 6 | 30 | . | 0 | . | . |
| Bot10 | 61 | endo-bot | 18 | 20 | 50 | 10 | 60.7 | 85.05 |

Bot = borderline ovarian tumours, fa = focal areas, endo = endometrioid, muc = mucinous,
ser = serous.

| EOC | age | type | stage | grade | VEGF (fa) | VEGF-C (%) | VEGFR-1 (%) | TP (%) | AVD x200 | HVD x200 |
|---|---|---|---|---|---|---|---|---|---|---|
| EOC1 | 48 | ser-eoc | . | 3 | . | . | . | . | 37.4 | 49.9 |
| EOC2 | 54 | unaccounted | . | 3 | 4 | . | . | . | 73.31 | 93.1 |
| EOC3 | 72 | unaccounted | 10 | 10 | 12 | . | . | . | 55.35 | 59.4 |
| EOC4 | 60 | ser-eoc | 3 | 3 | 4 | 10 | 70 | 30 | 21.6 | 32.4 |
| EOC5 | 79 | ser-eoc | 3 | 2 | 4 | 50 | . | 20 | 53.6 | 67.5 |
| EOC6 | 86 | muc-eoc | 3 | 10 | 0 | . | 40 | 40 | 32.4 | 37.8 |
| EOC7 | 65 | ser-eoc | 3 | 3 | 38 | 30 | 20 | 50 | 39.56 | 47.2 |
| EOC8 | 42 | ser-eoc | 3 | 3 | 2 | . | . | . | . | . |
| EOC9 | 67 | ser-eoc | 3 | 3 | 27 | 50 | 20 | . | 79.65 | 102.6 |
| EOC10 | 68 | endo-eoc | 3 | 3 | 16 | . | . | . | . | . |
| EOC11 | 54 | ser-eoc | 3 | 3 | 48 | 10 | 70 | . | 56.3 | 66.1 |
| EOC12 | 84 | endo-eoc | 1 | 2 | 15 | 20 | 30 | . | 69.26 | 101.2 |
| EOC13 | 82 | unaccounted | 10 | 3 | 28 | . | 70 | 20 | . | . |
| EOC14 | 54 | clear-cell | 10 | 10 | 10 | 20 | 70 | 20 | 30.51 | 39.1 |
| EOC15 | 48 | mixed | 3 | 3 | 3 | 50 | 50 | 80 | 18.36 | 22.9 |
| EOC16 | 74 | unaccounted | 3 | 3 | . | 10 | . | . | . | . |
| EOC17 | 61 | muc-eoc | 4 | 3 | 21 | 20 | . | . | . | . |
| EOC18 | 57 | ser-eoc | 3 | 3 | 1 | 30 | 20 | 80 | 33.75 | 37.8 |
| EOC19 | 65 | endo-eoc | 3 | 3 | 13 | . | 30 | 20 | 49.95 | 54 |
| EOC20 | 42 | clear-cell | 3 | 3 | 45 | 20 | . | . | . | . |
| EOC21 | 64 | muc-eoc | 4 | 2 | 12 | 0 | . | . | . | . |
| EOC22 | 41 | muc-eoc | 1 | 3 | 13 | 20 | 30 | . | 61.16 | 74.2 |
| EOC23 | 49 | ser-eoc | 3 | 2 | 58 | 0 | 20 | . | 58.05 | 71.5 |
| EOC24 | 74 | ser-eoc | 3 | 2 | 16 | 0 | 0 | . | 71.15 | 99.9 |
| EOC25 | 43 | ser-eoc | 3 | 2 | 10 | 10 | . | . | 28.35 | 32.4 |
| EOC26 | 74 | ser-eoc | 2 | 3 | 46 | . | . | . | . | . |
| EOC27 | 46 | ser-eoc | 3 | 1 | 24 | . | . | . | . | . |
| EOC28 | 56 | ser-eoc | 3 | 1 | 63 | 0 | . | . | . | . |
| EOC29 | 74 | ser-eoc | 3 | 2 | 5 | 20 | 50 | . | 40.1 | 48.6 |
| EOC30 | 65 | ser-eoc | 2 | . | 4 | 30 | . | 5 | 53.46 | 60.7 |
| EOC31 | 83 | clear-cell | 2 | 3 | 12 | 30 | 30 | 80 | 34.16 | 44.5 |
| EOC32 | 53 | clear-cell | 3 | 2 | 18 | 50 | 50 | . | . | . |
| EOC33 | 67 | ser-eoc | 3 | 2 | 33 | 60 | . | . | . | . |
| EOC34 | 41 | ser-eoc | 3 | 3 | 19 | 30 | . | . | . | 75.6 |
| EOC35 | 60 | ser-eoc | 3 | 10 | 10 | . | 50 | 70 | 32.81 | 37.8 |
| EOC36 | 36 | ser-eoc | 3 | 3 | 8 | 30 | . | 30 | 29.7 | 33.7 |
| EOC37 | 46 | ser-eoc | 3 | 3 | 31 | 20 | 50 | . | 44.55 | 55.3 |
| EOC38 | 42 | muc-eoc | 2 | 1 | 20 | 20 | 20 | . | 79.25 | 117.4 |
| EOC39 | 43 | ser-eoc | 3 | 3 | 67 | 60 | 0 | . | 30.65 | 32.4 |
| EOC40 | 57 | ser-eoc | 3 | 3 | 0 | 30 | . | 80 | 22.41 | 28.3 |
| EOC41 | 77 | mixed | 10 | 10 | 11 | 0 | 50 | 5 | 40.91 | 47.2 |
| EOC42 | 56 | ser-eoc | 3 | 3 | 10 | 20 | 40 | 80 | 43.2 | 47.2 |
| EOC43 | 57 | ser-eoc | 3 | 10 | 4 | 0 | 50 | 20 | 55.35 | 64.8 |
| EOC44 | 51 | ser-eoc | 3 | 3 | 8 | 50 | 50 | 80 | 10.26 | 13.5 |
| EOC45 | 68 | ser-eoc | 3 | 3 | 18 | 0 | 30 | 50 | 45.5 | 56.7 |
| EOC46 | 42 | ser-eoc | 1 | 2 | 7 | 30 | 30 | . | 56.7 | 72.9 |
| EOC47 | 70 | ser-eoc | 4 | 2 | . | 10 | . | . | . | . |
| EOC48 | 49 | muc-eoc | 3 | 1 | 4 | 30 | 0 | . | 43.61 | 47.2 |
| EOC49 | 25 | muc-eoc | 1 | 10 | 8 | 30 | 20 | 50 | 59.81 | 72.9 |
| EOC50 | 45 | ser-eoc | 3 | 2 | 12 | . | . | 5 | 73.31 | 81 |
| EOC51 | 77 | mixed | 3 | 3 | 10 | 20 | 50 | . | 48.2 | 64.8 |
| EOC52 | 67 | muc-eoc | 3 | 3 | 5 | 30 | 30 | 80 | 22.01 | 27 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| EOC53 | 42 | endo-eoc | 1 | 2 | 54 | 40 | 50 | 5 | 74.66 | 86.4 |
| EOC54 | 57 | ser-eoc | 3 | 1 | 4 | . | 40 | 0 | 40.5 | 51.3 |
| EOC55 | 73 | muc-eoc | 1 | 2 | 12 | 50 | . | . | 66.56 | 75.6 |
| EOC56 | 75 | endo-eoc | 3 | 2 | 3 | 10 | 50 | 20 | 22.95 | 29.7 |
| EOC57 | 39 | endo-eoc | 1 | 2 | 43 | 20 | 30 | 0 | 64.8 | 70.2 |
| EOC58 | 64 | endo-eoc | 1 | 2 | 17 | 40 | 20 | . | 36.05 | 45.9 |
| EOC59 | 62 | ser-eoc | 3 | 3 | . | 10 | . | . | . | . |
| EOC60 | 47 | muc-eoc | 3 | 3 | 53 | 0 | 40 | . | . | . |
| EOC61 | 78 | ser-eoc | 3 | 3 | 15 | 20 | . | . | . | . |
| EOC62 | 60 | muc-eoc | 1 | 1 | 25 | 30 | 50 | . | . | . |
| EOC63 | 57 | ser-eoc | 3 | 3 | 31 | 20 | 70 | . | 57.65 | 68.85 |
| EOC64 | 50 | endo-eoc | 3 | 2 | 20 | 50 | 50 | . | 63.05 | 68.85 |

EOC = epithelial ovarian cancers, fa = focal areas, endo = endometrioid, muc = mucinous,
ser = serous.

**Normal cases**

| NO | age | Type | IGF-1Ea | IGF-1Ec |
|---|---|---|---|---|
| NO3 | 45 | | neg | neg |
| NO4 | 25 | | neg | neg |
| NO5 | 51 | | neg | pos |
| NO7 | 47 | | pos | pos |
| NO8 | 59 | | neg | neg |
| NO10 | 57 | | pos | pos |
| NO11 | 49 | | neg | pos |
| NO12 | 48 | | neg | pos |
| NO13 | 54 | | neg | pos |
| NO15 | 40 | | pos | pos |
| NO16 | 43 | | neg | neg |
| NO17 | 62 | | neg | neg |
| NO18 | 48 | | neg | neg |
| NO19 | 44 | | neg | neg |
| NO20 | 44 | | pos | pos |
| NO21 | 46 | | pos | pos |
| NO22 | 81 | | pos | pos |
| NO23 | 49 | | neg | pos |

**Cystadenomas**

| | | | | |
|---|---|---|---|---|
| Cy31 | 46 | other | pos | pos |
| Cy29 | 46 | mucinous | pos | pos |
| Cy1 | 34 | mucinous | pos | pos |
| Cy19 | 65 | mucinous | pos | pos |
| Cy2 | 24 | mucinous | neg | pos |
| Cy3 | 45 | serous | neg | neg |
| Cy4 | 44 | mucinous | pos | pos |
| Cy5 | 32 | serous | pos | pos |
| Cy21 | 32 | other | pos | pos |
| Cy6 | 61 | serous | neg | pos |
| Cy7 | 37 | mucinous | pos | pos |
| Cy22 | 54 | other | pos | pos |
| Cy24 | 37 | other | pos | pos |

**EOC cases**

| age | type | stage | grade | IGF-1Ea | IGF-1Ec |
|---|---|---|---|---|---|
| 61 | endometrioid | 1 | 1 | pos | pos |
| 52 | mucinous | . | 1 | pos | pos |
| 54 | clear cell | . | . | pos | pos |
| 48 | serous | 4 | 3 | pos | pos |
| 54 | . | . | . | pos | neg |
| 41 | mucinous | 1 | 3 | pos | pos |
| 49 | serous | 3 | 2 | pos | pos |
| 74 | serous | 3 | 2 | pos | pos |
| 43 | serous | 3 | 2 | pos | pos |
| 74 | serous | 3 | 2 | pos | pos |
| 65 | serous | 2 | . | pos | pos |
| 41 | serous | 3 | 3 | pos | pos |
| 79 | serous | 3 | 2 | pos | pos |
| 60 | serous | 3 | . | pos | pos |
| 43 | serous | 3 | 3 | pos | pos |
| 57 | serous | 3 | 3 | pos | pos |
| 77 | . | . | . | pos | pos |
| 29 | serous | 3 | . | pos | pos |
| 56 | serous | 3 | 3 | pos | pos |
| 57 | serous | 3 | . | pos | pos |
| 42 | serous | 1 | 2 | pos | pos |
| 70 | serous | 4 | 2 | pos | pos |
| 49 | mucinous | 3 | 1 | pos | pos |
| 86 | mucinous | 3 | . | pos | pos |
| 65 | serous | 3 | 3 | pos | pos |
| 25 | mucinous | 1 | . | pos | pos |
| 55 | mucinous | 3 | 3 | pos | pos |
| 45 | serous | 3 | 2 | pos | pos |
| 77 | . | 3 | 3 | pos | pos |
| 67 | mucinous | 3 | 3 | pos | pos |
| 73 | . | 1 | 2 | pos | pos |
| 39 | endometrioid | 1 | 2 | pos | pos |
| 54 | serous | 3 | 3 | pos | pos |

| | | | | |
|---|---|---|---|---|
| Cy10 | 47 | mucinous | neg | pos |
| Cy12 | 31 | serous | pos | pos |
| Cy13 | 56 | mucinous | pos | pos |
| Cy14 | 50 | mucinous | pos | pos |
| Cy15 | 33 | mucinous | pos | pos |
| Cy16 | 39 | serous | pos | pos |
| Cy27 | 48 | other | pos | pos |
| Cy17 | 75 | mucinous | pos | pos |
| Cy18 | 51 | mucinous | pos | pos |
| Cy30 | 36 | other | pos | pos |

| | | | | | |
|---|---|---|---|---|---|
| 64 | endometrioid | 1 | 2 | pos | pos |
| 47 | mucinous | 3 | 3 | pos | pos |
| 78 | serous | 3 | 3 | pos | pos |
| 55 | endometrioid | 1 | 2 | pos | pos |
| 57 | serous | 3 | 3 | pos | pos |
| 67 | serous | 3 | 3 | pos | pos |

## PATIENTS' DETAILS AND ELISA RESULTS

| Case | week | sVEGF | sVEGFR-2 |
|---|---|---|---|
| HV1 | 1 | 252.4 | |
| HV2 | 1 | 358.9 | |
| HV3 | 1 | 578.8 | |
| HV4 | 1 | 516.8 | |
| HV5 | 1 | 98.4 | |
| HV6 | 1 | 505 | |
| HV7 | 1 | 380.8 | |
| HV8 | 1 | 739.5 | |
| HV9 | 1 | 606.6 | |
| HV10 | 1 | 573.6 | |
| HV11 | 1 | 647.3 | |
| HV12 | 1 | 432.4 | |
| HV13 | 1 | 330.4 | |
| HV14 | 1 | 254.4 | 10559 |
| HV15 | 1 | 658.1 | |
| HV16 | 1 | 164.8 | 7342.5 |
| HV17 | 1 | 87.2 | |
| HV18 | 1 | 143.4 | 8048 |
| HV19 | 1 | 401.3 | |
| HV20 | 1 | 467.4 | |
| HV21 | 1 | 220.5 | 9439 |
| HV22 | 1 | 478.2 | |
| HV23 | 1 | 352.4 | |
| HV24 | 1 | 445 | 8291.5 |
| HV25 | 1 | 358.6 | |
| HV26 | 1 | 294.2 | |
| HV27 | 1 | 131.8 | |
| HV28 | 1 | 225.8 | |
| HV29 | 1 | 323.4 | |
| HV30 | 1 | 465.1 | |
| HV31 | 1 | 152.2 | |
| HV32 | 1 | 358.8 | 9991.5 |
| HV33 | 1 | 272.9 | 12072 |
| HV34 | 1 | 201 | |
| HV35 | 1 | 56.9 | |
| HV36 | 1 | 368.8 | |
| HV37 | 1 | 204.3 | |

| case | week | sVEGF | sVEGFR-2 |
|---|---|---|---|
| HV1 | 2 | 442.7 | |
| HV3 | 2 | 614 | |
| HV4 | 2 | 358.6 | |
| HV6 | 2 | 408.9 | |
| HV7 | 2 | 324 | |
| HV11 | 2 | 677.5 | |
| HV13 | 2 | 386.9 | |
| HV14 | 2 | 844.7 | 9351 |
| HV16 | 2 | 194.4 | 8079 |
| HV18 | 2 | 59.8 | 8119.5 |
| HV21 | 2 | 115.1 | 9777.5 |
| HV24 | 2 | 614.4 | 9271 |
| HV15 | 2 | 943.2 | |
| HV25 | 2 | 571.4 | |
| HV26 | 2 | 322.2 | |
| HV27 | 2 | 180 | |
| HV28 | 2 | 248.6 | |
| HV29 | 2 | 355.9 | |
| HV31 | 2 | 120.8 | |
| HV32 | 2 | 718.6 | 10005 |
| HV33 | 2 | 237.2 | 11265.5 |
| HV34 | 2 | 189.1 | |
| HV37 | 2 | 333.4 | |
| HV38 | 2 | 228.3 | |
| HV42 | 2 | 1429.7 | |
| HV39 | 2 | 89.3 | 11706 |
| HV40 | 2 | 350.1 | |
| HV41 | 2 | 358.5 | |
| HV43 | 2 | 867.9 | |

| | | | |
|---|---|---|---|
| HV38 | 1 | 1779.2 | |
| HV39 | 1 | 188.5 | 11976.5 |
| HV40 | 1 | 270 | |
| HV41 | 1 | 373.4 | |
| HV42 | 1 | 618.9 | |
| HV43 | 1 | 159.1 | |
| HV44 | 1 | 436.5 | |

| Case | week | sVEGF | sVEGFR-2 |
|---|---|---|---|
| HV1 | 3 | 304.4 | |
| HV3 | 3 | 640.4 | |
| HV4 | 3 | 500.8 | |
| HV7 | 3 | 314 | |
| HV11 | 3 | 456.4 | |
| HV13 | 3 | 453.5 | |
| HV16 | 3 | 158.3 | 7290.5 |
| HV17 | 3 | 52.7 | |
| HV18 | 3 | 144.1 | 7849.5 |
| HV21 | 3 | 201.1 | 9976.5 |
| HV26 | 3 | 216.9 | |
| HV28 | 3 | 264.1 | |
| HV29 | 3 | 540.4 | |
| HV30 | 3 | 244.2 | |
| HV31 | 3 | 197.6 | |
| HV32 | 3 | 550.3 | |
| HV37 | 3 | 220.1 | |
| HV38 | 3 | 431.5 | |
| HV39 | 3 | 170.3 | 11754 |
| HV40 | 3 | 504.3 | |
| HV43 | 3 | 1021.4 | |

| case | week | sVEGF | sVEGFR-2 |
|---|---|---|---|
| HV3 | 4 | 638.4 | |
| HV4 | 4 | 1121 | |
| HV7 | 4 | 322 | |
| HV16 | 4 | 145.1 | |
| HV17 | 4 | 91.5 | |
| HV18 | 4 | 142.1 | 8497 |
| HV21 | 4 | 145 | 10086.5 |
| HV26 | 4 | 265.6 | |
| HV28 | 4 | 272.2 | |
| HV29 | 4 | 378.2 | |
| HV31 | 4 | 174.2 | |
| HV39 | 4 | 182.9 | |
| HV40 | 4 | 270.2 | |

HV = healthy volunteers, sVEGF = serum VEGF levels, sVEGFR-2 = serum VEGFR-2 levels.

| Case | age | sVEGF | sVEGFR-2 | Platelet count |
|---|---|---|---|---|
| NO1 | 51 | 164.9 | 6390.5 | 207 |
| NO2 | 46 | 172.6 | 4547 | 281 |
| NO3 | 45 | 48 | 10815.2 | 261 |
| NO4 | 25 | 59 | 11893 | 179 |
| NO5 | 51 | 135.9 | 6912.5 | . |
| NO6 | 38 | 374.4 | 5546.5 | 407 |
| NO7 | 47 | 198.1 | 8948.5 | 518 |
| NO8 | 59 | 479.5 | 8287 | . |
| NO9 | 74 | 89.4 | 7937 | 231 |
| NO10 | 57 | 313.1 | 9287.5 | . |
| NO11 | 49 | 1425.2 | 8435 | 477 |
| NO12 | 48 | 257.2 | 11895.5 | 344 |
| NO13 | 54 | 142.6 | 8596.5 | 191 |
| NO14 | 50 | 277.2 | 7859.5 | . |
| NO15 | 40 | 98.8 | . | 212 |
| NO16 | 43 | 88.4 | 6048 | 208 |
| NO19 | 44 | 544.9 | . | . |

| | | | | |
|---|---|---|---|---|
| NO17 | 62 | 727.9 | 5086.5 | 269 |
| NO18 | 48 | 272.9 | 9778.5 | 256 |
| NO20 | 44 | 571.4 | 7401.5 | . |
| NO25 | 44 | 171.9 | 12123 | . |
| NO21 | 46 | 167.4 | 6706 | . |
| NO22 | 81 | 448.1 | 6665 | . |
| NO23 | 49 | 542 | 11670.5 | . |
| NO24 | 46 | 709.4 | 8468.5 | . |

NO = normal ovarian tissue (or controls), sVEGF = serum VEGF levels, sVEGFR-2 = serum VEGFR-2 levels.

| Case | age | subtype | sVEGF | sVEGFR-2 | platelet count |
|---|---|---|---|---|---|
| Cy28 | 26 | mucinous | 213.6 | 11614.5 | |
| Cy29 | 46 | mucinous | 713.9 | 6963 | 221 |
| Cy1 | 34 | mucinous | 77.9 | 9001.5 | 180 |
| Cy19 | 65 | mucinous | 264.3 | 6276 | 238 |
| Cy2 | 24 | mucinous | 106.6 | 6293 | . |
| Cy20 | 41 | mucinous | 617.3 | 8535 | 297 |
| Cy3 | 45 | serous | 520 | 7655.5 | 270 |
| Cy4 | 44 | mucinous | 78.4 | 13410 | 198 |
| Cy5 | 32 | serous | 184.9 | 7137 | . |
| Cy21 | 32 | unclassified | 359.4 | 9500 | . |
| Cy6 | 61 | serous | 168.4 | 5914 | 14.2 |
| Cy7 | 37 | mucinous | 177.5 | 6502 | 285 |
| Cy8 | 38 | serous | 309 | 9435 | . |
| Cy9 | 59 | mucinous | 927.9 | 8104.5 | 289 |
| Cy22 | 54 | unclassified | 351.9 | 4308 | . |
| Cy23 | 51 | mucinous | 439.7 | 9756 | 347 |
| Cy24 | 37 | unclassified | 200.3 | 10691 | 357 |
| Cy25 | 52 | unclassified | 344.7 | 7303.5 | 304 |
| Cy10 | 47 | mucinous | 141.9 | 8214.5 | 231 |
| Cy12 | 31 | serous | 217.6 | 7603.5 | 330 |
| Cy13 | 56 | mucinous | 1127.8 | 9012 | 322 |
| Cy14 | 50 | mucinous | 189.5 | 5687 | 148 |
| Cy15 | 33 | mucinous | 248.3 | 15007 | 297 |
| Cy16 | 39 | serous | 45.8 | 11492 | 226 |
| Cy26 | 52 | serous | 302.3 | 5734.5 | |
| Cy27 | 48 | unclassified | 17.6 | 9224.5 | 71 |
| Cy17 | 75 | mucinous | 411 | 6843.5 | 200 |
| Cy18 | 51 | mucinous | 130.4 | . | 286 |
| Cy30 | 36 | unclassified | 289.2 | 12977 | 200 |

Cy = ovarian cystadenomas, sVEGF = serum VEGF levels, sVEGFR-2 = serum VEGFR-2 levels.

| Case | age | subtype | stage | grade | sVEGF | sVEGFR-2 | platelet count |
|---|---|---|---|---|---|---|---|
| EOC65 | 52 | mucinous | . | 1 | 771.2 | 5318 | . |
| EOC66 | 54 | clear cell | . | . | 745.4 | 9284.5 | . |
| EOC20 | 42 | clear cell | III | 3 | 313.2 | 9146.5 | . |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| EOC15 | 48 | mixed | III | 3 | 394.8 | 5526 | 309 |
| EOC 67 | 43 | serous | III | 2 | 278 | 8647 | . |
| EOC 68 | 57 | serous | III | 1 | 224.5 | 8800.5 | . |
| EOC 69 | 46 | serous | III | 1 | . | 7628.5 | . |
| EOC 70 | 35 | mucinous | II | . | 685.7 | 7898 | 324 |
| EOC30 | 65 | serous | II | . | 483 | 6779 | . |
| EOC31 | 83 | clear cell | II | 3 | 2109.7 | 7058 | . |
| EOC 71 | 45 | mucinous | II | . | 378 | 13318.5 | . |
| EOC 72 | 67 | serous | III | 3 | 933.5 | 6447 | . |
| EOC 73 | 67 | serous | III | 2 | 1277 | 7999 | . |
| EOC5 | 79 | serous | III | 2 | 310 | 8210 | . |
| EOC 74 | 36 | serous | III | 3 | 792.7 | 9481 | . |
| EOC 75 | 62 | serous | III | 3 | 598.1 | 11078.5 | . |
| EOC 76 | 61 | mucinous | IV | 3 | 714.4 | . | . |
| EOC 77 | . | unclassified | . | . | 82.1 | 1266.5 | . |
| EOC 78 | 57 | serous | III | 3 | 1960.7 | . | . |
| EOC4 | 60 | serous | III | 3 | 1236.2 | 8089 | . |
| EOC79 | 29 | serous | III | . | . | 5435.5 | 387 |
| EOC80 | 56 | serous | III | 3 | 622.4 | 7019.5 | . |
| EOC43 | 57 | serous | III | . | 568.1 | 7946.5 | . |
| EOC44 | 51 | serous | III | 3 | 573 | 5064 | . |
| EOC81 | 45 | mixed | . | . | 342 | 9869.5 | . |
| EOC6 | 86 | mucinous | III | . | 1175.9 | 4231 | . |
| EOC7 | 65 | serous | III | 3 | 1432.7 | 8380.5 | . |
| EOC82 | 25 | mucinous | I | . | 206.8 | 7171 | 476 |
| EOC83 | 55 | mucinous | III | 3 | 322.3 | 12980.5 | . |
| EOC84 | . | unclassified | . | . | 368.8 | 11357.5 | . |
| EOC52 | 67 | mucinous | III | 3 | 416.9 | 6624.5 | . |
| EOC85 | 59 | serous | III | 2 | 1072.2 | 10338.5 | . |
| EOC54 | 57 | serous | III | 1 | 30.4 | 3132.5 | . |
| EOC56 | 75 | endometrioid | III | 2 | 812.6 | 5400 | . |
| EOC60 | 47 | mucinous | III | 3 | 644.8 | 6940 | . |
| EOC86 | 75 | mucinous | I | . | 338.1 | 8345 | 279 |
| EOC40 | 57 | serous | III | 3 | 1883.3 | 5918 | . |

EOC = epithelial ovarian cancers, sVEGF = serum VEGF levels, sVEGFR-2 = serum VEGFR-2 levels.

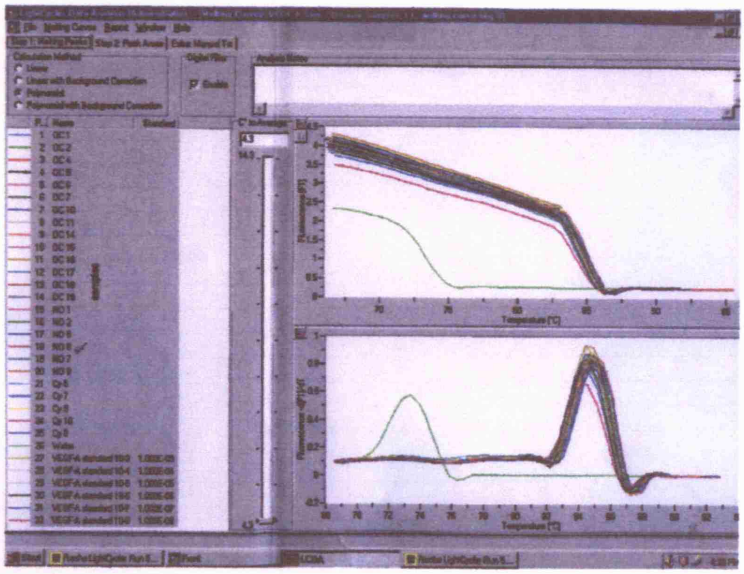
## PATIENTS' DETAILS AND mRNA RESULTS

| Case | VEGF mRNA | IGF-1Ec mRNA | IGF-1Ea mRNA |
|---|---|---|---|
| mRNA-Ca1 | 0.000000544 | 5.83E-08 | 0.00037 |
| mRNA-Ca2 | 0.00000221 | 0.000000225 | 0.000433 |
| mRNA-Ca3 | 0.000000534 | 0.000000682 | 0.000172 |
| mRNA-Ca4 | 0.000000407 | 2.45E-08 | 0.00109 |
| mRNA-Ca5 | 0.000000112 | 1.37E-08 | 0.000136 |
| mRNA-Ca6 | 0.000000175 | 2.9E-09 | 0.0000154 |
| mRNA-Ca7 | 0.000000165 | 0.00000106 | 0.000001 |
| mRNA-Ca8 | 0.0000121 | 0.000000441 | 0.00000162 |
| mRNA-Ca9 | 0.00000311 | 0.000000341 | 0.00000109 |
| mRNA-Ca10 | 0.000000926 | 0.000000033 | 0.000000741 |
| mRNA-Ca11 | 0.00000269 | 1.8E-10 | 0.0000007 |
| mRNA-Ca12 | 0.000000026 | 1.93E-09 | 0.000000592 |
| mRNA-Ca13 | 0.00000567 | 5.02E-08 | . |

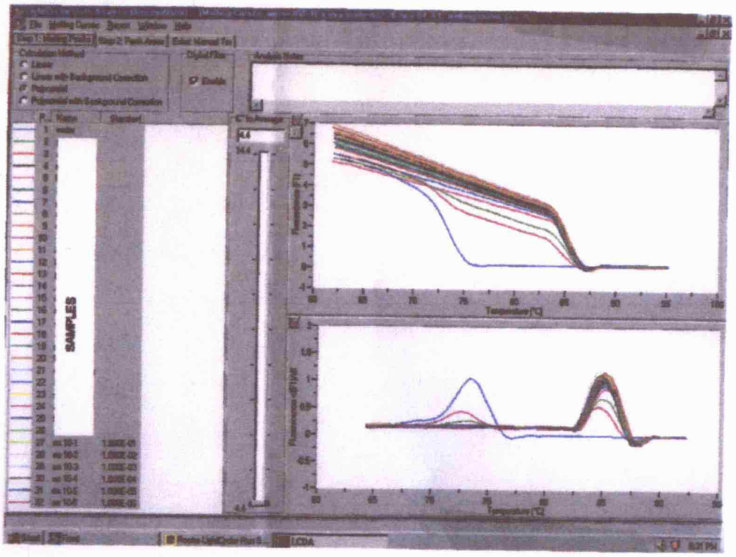
| | | | |
|---|---|---|---|
| mRNA-Ca14 | 0.00000301 | 1.7E-10 | . |
| mRNA-Ca15 | 0.000000017 | 0.00000136 | . |
| mRNA-Ca16 | 0.000000205 | 2.87E-09 | . |
| mRNA-Ca17 | 0.000000026 | 0.000000117 | . |
| mRNA-Ca18 | 0.000000047 | 0.00000133 | . |
| mRNA-Ca19 | 0.0000143 | 2.18E-08 | . |
| mRNA-Ca20 | 0.000000225 | . | . |
| mRNA-Ca21 | 0.00000005 | . | . |
| mRNA-Cy1 | 0.000000494 | 0.00000012 | 0.000618 |
| mRNA-Cy2 | 0.00000229 | 0.000000182 | 0.00119 |
| mRNA-Cy3 | 0.00000901 | 2.76E-08 | 0.00195 |
| mRNA-Cy4 | 0.000000057 | 1.76E-08 | 0.0000705 |
| mRNA-Cy5 | 0.000000036 | 1.06E-09 | 0.0000352 |
| mRNA-Cy6 | 0.00000282 | 3.2E-10 | 0.00000275 |
| mRNA-Cy7 | 0.000000012 | 0.000000682 | 0.000000806 |
| mRNA-Cy8 | 0.00000118 | 0.000000574 | 0.000328 |
| mRNA-Cy9 | 0.0000025 | 0.00000163 | 0.00000661 |
| mRNA-Cy10 | 0.00000246 | 3.83E-09 | 0.000312 |
| mRNA-Cy11 | 0.000000437 | 0.00000414 | 0.000497 |
| mRNA-Cy12 | 0.000000023 | 0.00000316 | 0.00000148 |
| mRNA-Cy13 | 0.000000006 | 0.000000343 | 0.00133 |
| mRNA-Cy14 | 0.000000016 | 5.21E-09 | 0.00027 |
| mRNA-Cy15 | 0.000000024 | 0.0000015 | 0.00017 |
| mRNA-Cy16 | 0.000000142 | 4.63E-08 | 0.00000149 |
| mRNA-Cy17 | 0.000000823 | 1.04E-08 | 0.00000115 |
| mRNA-Cy18 | 0.000000175 | 0.00000229 | 0.00000902 |
| mRNA-Cy19 | 0.00000208 | 0.00000747 | 0.00252 |
| mRNA-Cy20 | 0.000000621 | 0.00000128 | 0.000149 |
| mRNA-Cy21 | 0.00000761 | . | 0.0000265 |
| mRNA-Cy22 | . | . | 0.000377 |
| mRNA-Cy23 | . | . | 0.0021 |
| mRNA-Cy24 | . | . | 0.00106 |
| mRNA-No1 | 0.000000163 | 2.55E-08 | 0.00000105 |
| mRNA-No2 | 0.00000151 | 1.27E-09 | 0.00000244 |
| mRNA-No3 | 0.000000017 | 7.62E-09 | 0.00000167 |
| mRNA-No4 | 0.000000332 | 0.000000139 | 0.00152 |
| mRNA-No5 | 0.00000341 | 1.66E-08 | 0.0000015 |
| mRNA-No6 | 0.000000117 | 0.000000568 | 0.000427 |
| mRNA-No7 | 0.000000697 | 0.0000014 | 0.000361 |
| mRNA-No8 | 0.00000189 | 3.61E-08 | 0.0000026 |
| mRNA-No9 | 0.00000267 | 0.00000105 | 0.00461 |
| mRNA-No10 | 0.000000194 | 0.000000118 | 0.000479 |
| mRNA-No11 | 0.00000025 | 0.000000369 | 0.000891 |
| mRNA-No12 | 0.0000014 | 4.48E-08 | 0.000269 |
| mRNA-No13 | 0.00000156 | 0.00000028 | 0.000745 |
| mRNA-No14 | 0.000000675 | 0.000000118 | 0.0005 |
| mRNA-No15 | 0.00000328 | 0.000000283 | 0.000785 |
| mRNA-No16 | 0.00000268 | 4.96E-08 | 0.000654 |
| mRNA-No17 | 0.000000056 | 2.65E-08 | 0.000112 |
| mRNA-No18 | 0.000000038 | 0.00000041 | 0.00117 |
| mRNA-No19 | 0.000001 | 0.000000263 | 0.00000343 |
| mRNA-No20 | . | 5.46E-08 | 0.00167 |
| mRNA-No21 | . | 0.00000903 | . |

Ca = Cancer, Cy = cystadenoma, No = Normal ovary.

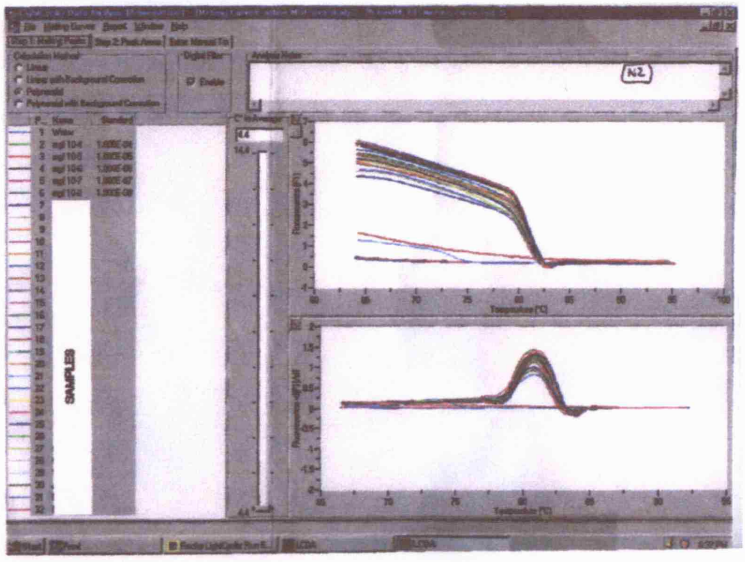
## MELTING CURVES



VEGF

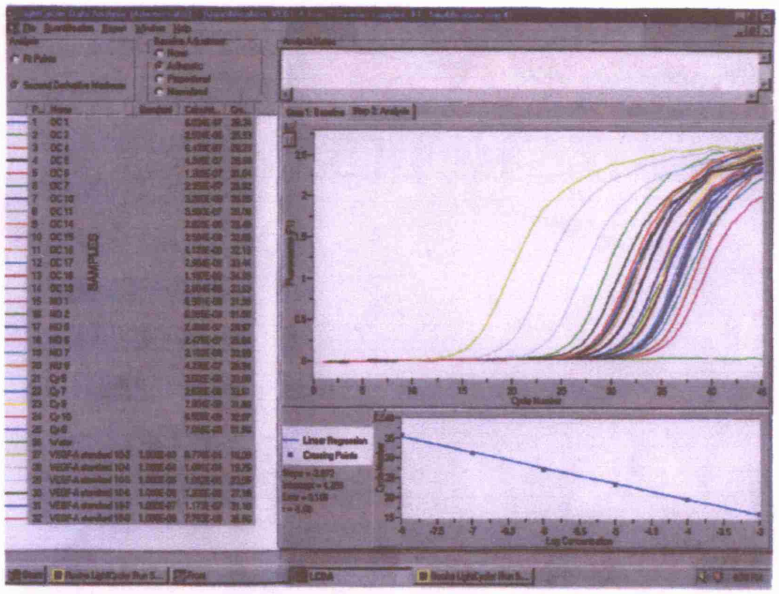


IGF-1Ea



IGF-1Ec

## STANDARD CURVES



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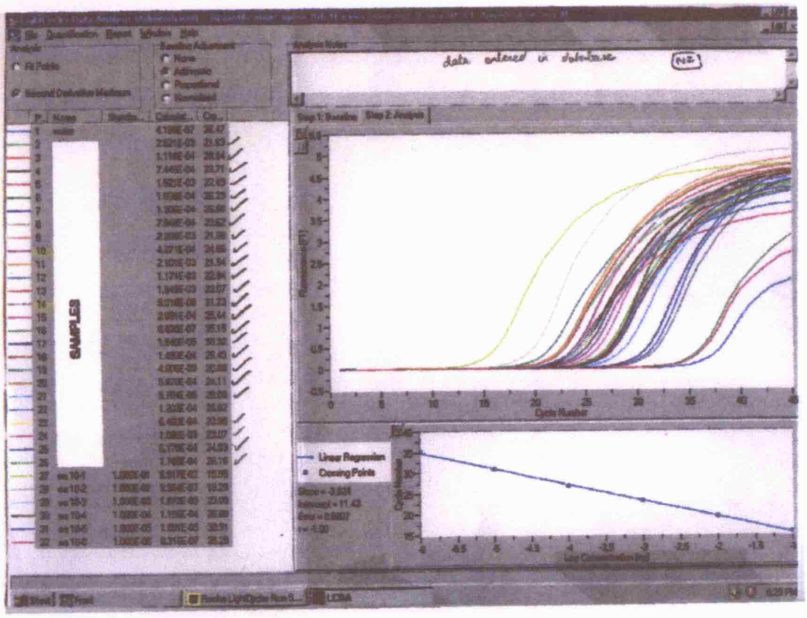

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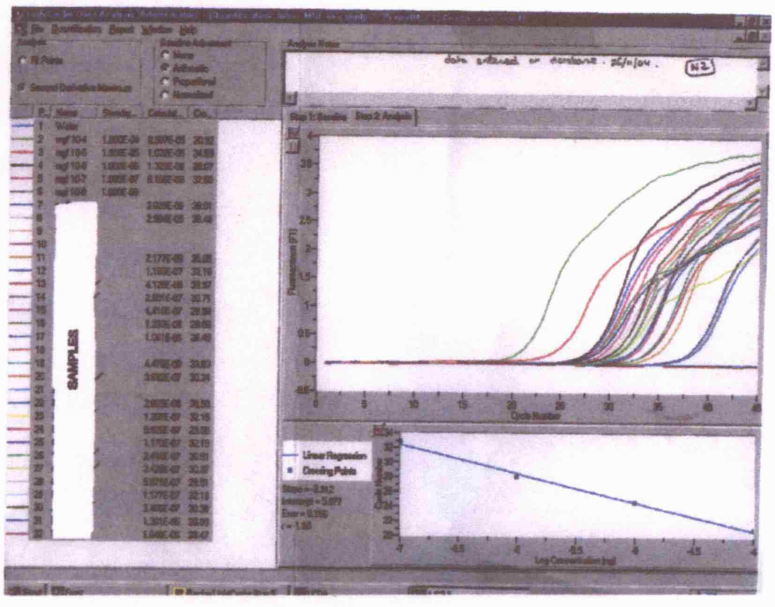

IGF-IEc

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## CONTRIBUTIONS TO OTHER PUBLICATIONS

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